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 ($O_2^-$) 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$_2$O$_2$ and hydrogen sulfide (H$_2$S), 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$_2^-$ 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$_2^-$ is reduced, lowering their significance in future cell signalling pathways. In a similar manner, NO and H$_2$S 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$_2$O$_3$
may interfere with the measure of NO\textsuperscript{•} 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\textsuperscript{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 H₂S 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
of NO metabolism changes in cells needs to be had, with small changes perhaps not having the effects that might be assumed.

**Considerations of redox chemistry**

Probably the first consideration of how nitric oxide impacts on redox is to determine which species is present. Nitric oxide chemistry discussions need to include the radical form NO\(^{\cdot}\), but also species such as nitroxyl (NO\(^{-}\)) and nitrosonium (NO\(^{+}\)) ions (Lancaster Jr, 2015). The chemistry of these three nitrogen species is quite different and methods to supply NO to experimental systems may deliver one of these (Hughes, 1999), not necessarily NO\(^{\cdot}\) as might be wanted or expected.

Despite the interaction of NO with redox, the redox status of the cell is important to determine. Often the overall redox of the cell is estimated by the measurement of glutathione (reviewed by Schafer and Buettner, 2001). It is not sufficient to simply measure the concentrations of the reduced form GSH, but the concentration of the oxidised form, GSSG, also needs to be determined. These two pieces of data can then be used to calculate the total amount of glutathione present, as well as the oxidised:reduced ratio (ox:red ratio).

To determine the redox status of the experimental solution being studied, \(E_h\), there are two numbers which are needed. It is important to know the ox:red ratio of the chemical species under study, but the mid-point potential of the species is also needed, that is the \(E_m\). Once these numbers are known they can be used to calculate \(E_h\) using the Nernst Equation, as shown in Equation 1.

**Equation 1: The Nernst Equation (redox equation)** assuming an intracellular pH of 7.4.
\[ E_h = E_{m(pH7.4)} + \frac{RT \times 2.303 \log [\text{oxidised}]}{nF} \frac{\text{[reduced]}}{\text{[reduced]}} \]

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.

\[ 2\text{GSH} \rightarrow \text{GSSG} \]

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 \( \text{H}_2\text{S} \), 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:**

\[
\text{Redox environment} = \sum_{i=1}^{n} E_i \times [\text{reduced species}]_i
\]

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 \( \gamma \)-glutamyl-cysteine (\( \gamma \)-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 H₂S 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 (H₂O₂). 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 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⁻/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⁻ 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$_2$S couple would not favour the H$_2$S 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$_2^-$ and H$_2$S, 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 H₂S (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 H₂S. 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.

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Zweier JL, Samouilov A, Kuppusamy P. 1999, Non-enzymatic nitric oxide synthesis in biological systems. Biochimica et Biophysica Acta. 1411, 250-262
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$_2$O$_2$ and H$_2$S.
Figure 2: Reactions in which NO may partake in biological systems.

\[
\begin{align*}
O_2^- + NO^- & \rightarrow ONOO^- \quad \text{(peroxynitrite)} \\
2NO^- + O_2 & \rightarrow 2NO_2^- \quad \text{(nitrogen dioxide)} \\
2NO_2^- + 2NO^- & \rightarrow 2N_2O_3 \quad \text{(dinitrogen trioxide)} \\
NO^- + GSH & \rightarrow GSNO \quad \text{(S-nitrosoglutathione)} \\
NO^- + H_2S & \rightarrow HSNO \quad \text{(nitrosothiol)}
\end{align*}
\]
<table>
<thead>
<tr>
<th>Source of NO</th>
<th>Comment</th>
<th>Reference(s)</th>
</tr>
</thead>
</table>
| 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) |
Table 2: Methods for measuring NO in plants.

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

<table>
<thead>
<tr>
<th>Method reported</th>
<th>Comments</th>
<th>Reference for use of method</th>
</tr>
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<tbody>
<tr>
<td>Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR)</td>
<td>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.</td>
<td>Weaver et al. (2005) Bright et al. (2009) Galatro and Puntarulo (2016)</td>
</tr>
<tr>
<td>Oxyhaemoglobin assay</td>
<td>Can suffer interference from ROS. Detection limit of 1.3-2.8 nM.</td>
<td>Murphy and Noack (1994)</td>
</tr>
<tr>
<td>Diaminofluorescein-based fluorescence</td>
<td>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.</td>
<td>Kojima et al. (1998) Planchet and Kaiser (2006) Yang et al. (2014)</td>
</tr>
<tr>
<td>Griess assay</td>
<td>Background nitrite/nitrate levels, which may be high in plants, can cause quantification problems. Sensitivity originally reported to be 0.5 µM.</td>
<td>Vitecek et al. (2008)</td>
</tr>
<tr>
<td>Ozone-chemiluminescence</td>
<td>Detection limits may be as low as 20-50 pmol.</td>
<td>Byrnes et al. (1996) Rumer et al. (2009)</td>
</tr>
<tr>
<td>NO electrodes/meter</td>
<td>Useful for extracellular or in vitro work. Often difficult for in vivo work.</td>
<td>Arasimowicz et al. (2009)</td>
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<td>Mass spectrometry</td>
<td>Can be used to detect N isotopes.</td>
<td>Conrath et al. (2004)</td>
</tr>
<tr>
<td>NOS-like activity</td>
<td>Often based on citrulline production these assays may be measuring argininosuccinate lyase activity.</td>
<td>Tischner et al. (2007)</td>
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