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REVIEW PAPER

Current approaches to measure nitric oxide in plants

Abhaypratap Vishwakarma[1,*], Aakanksha Wany[1,*], Sonika Pandey[1,*], Mallesham Bulle[1,*], Aprajita Kumari[1], Reddy Kishorekumar[1], Abir U. Igamberdiev[2], Luis A.J. Mur[3] and Kapuganti Jagadis Gupta[1,†]

[1] National Institute of Plant Genome Research, Aruna Asaf Ali Marg, 110067, New Delhi, India
[2] Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada
[3] Institute of Environmental and Rural Science, Aberystwyth University, Edward Llwyd Building, Aberystwyth SY23 3DA, UK

* These authors contributed equally to this work.
† Correspondence: jgk@nipgr.ac.in

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Abstract

Nitric oxide (NO) is now established as an important signalling molecule in plants where it influences growth, development, and responses to stress. Despite extensive research, the most appropriate methods to measure and localize these signalling radicals are debated and still need investigation. Many confounding factors such as the presence of other reactive intermediates, scavenging enzymes, and compartmentation influence how accurately each can be measured. Further, these signalling radicals have short half-lives ranging from seconds to minutes based on the cellular redox condition. Hence, it is necessary to use sensitive and specific methods in order to understand the contribution of each signalling molecule to various biological processes. In this review, we summarize the current knowledge on NO measurement in plant samples, via various methods. We also discuss advantages, limitations, and wider applications of each method.

Keywords: Chemiluminiscence, haemoglobin, mitochondria, nitric oxide, quantum cascade laser, redox.

Introduction

The gaseous free radical nitric oxide (NO) emerged as an important signalling molecule in plants. This versatile molecule plays various roles in plants during various developmental stages (seed germination, root growth, flowering, growth of pollen tube, and leaf senescence), stem cell regulation, stomatal movement, regulation of primary metabolism, and protection against biotic/abiotic stresses (Besson-Bard *et al.*, 2008*b*; Wilson *et al.*, 2008; Yu *et al.*, 2014; Domingos *et al.*, 2015; Fancy *et al.*, 2017).

In the case of animals, NO acts as a potent endogenous vasodilator and plays important roles in inflammation, inhibition of platelet aggregation, regulation of immune responses, cGMP signalling, and protection during ischaemia and reperfusion injury (Schmidt and Walter, 1994; Phillips *et al.*, 2009). In animals, the majority of NO is produced by a family of nitric oxide synthases (NOSs). NOS catalyses deamination of arginine to citrulline. Three NOS enzymes are characterized in animal systems, namely endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS). NOS has homology to P450 cytochrome *c* reductases. NOS activity requires various substrates and cofactors such as NADPH, FMN, FAD, tetrahydrobiopterin (BH_4), and calcium/calmodulin. In addition to NOS, mitochondria of animal system are also known to produce NO via a nitrite-dependent pathway (Shiva, 2010).

Comparison of NO signalling in animals and plants revealed several commonalities (Wendehenne *et al.*, 2001). Plant

NO signalling operates via the *S*-nitrosylation process and also via activation of calcium, protein kinase cGMP signalling, and reactive oxygen species (ROS) (Wendehenne *et al.*, 2001; Gaupels *et al.*, 2011). Despite several attempts to identify NOS, a clear picture of its existence in land plants has not been obtained so far. However, the existence of NOS-like activity has been shown in various species and also in response to stress (reviewed in Corpas *et al.*, 2009). The conclusions on NOS activity are mainly based on two methods, one is the inhibition of NO production in response to L-arginine analogues such as AET [2-(2-aminoethyl)isothiourea], PBITU [*S*,*S*′-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea], L-NIL [N^6-(1-iminoethyl)- L-lysine], L-NAME (N^G-nitro-L-arginine methyl ester), and L-NMMA (N^G-monomethyl-L-arginine), and the second is the arginine/citrulline assay. The latter has recently been questioned because argininosuccinate, synthesized as a side reaction from arginine and fumarate by argininosuccinate lyase, can give false-positive results (Tischner *et al.*, 2007). Recent evidence suggests that a few algal species such as *Ostreococcus tauri* possess NOS (Foresi *et al.*, 2010; Santolini *et al.*, 2017).

NO is also produced via nitrate reductase (NR) by utilizing nitrite as an ultimate substrate in an NAD(P)H-dependent reaction (Rockel *et al.*, 2002; Planchet *et al.*, 2005). NR contains molybdenum in its active site, and the enzyme activity is inhibited by tungstate which is an antagonist of molybdate (Bright *et al.*, 2006). In Arabidopsis, NR is encoded by two genes *NIA1* and *NIA2*. Deletion of *NIA2* in Arabidopsis resulted in only 10% NR activity compared with wild-type plants (Wilkinson and Crawford, 1993). NR-dependent NO increases in light and dark with anoxia treatment (Planchet *et al.*, 2005). In addition, mitochondria can reduce nitrite to NO under low oxygen conditions using nitrite as substrate (Gupta *et al.*, 2005; Vishwakarma *et al.*, 2018); this occurs at the site of complex III, IV, and alternative oxide (AOX) (Gupta *et al.*, 2018). In addition, NO may also be synthesized by a plasma membrane-bound nitrite-NO oxidoreductase (Ni-NOR). However, it was found to be root specific and acts as a sensor of nitrate availability in the soil (Stohr and Stremlau, 2006). However, other reductive pathways for NO synthesis operating via xanthine dehydrogenase/oxidase where nitrite acts as a substrate (Godber *et al.*, 2000) are also present. Plants also produce NO via polyamine- and hydroxylamine-mediated pathways. These pathways are still poorly characterized and need further investigation (Tun *et al.*, 2006; Rumer *et al.*, 2009).

Crucially, NO rapidly reacts with O_2^-, leading to formation of $ONOO^-$. The production of $ONOO^-$ takes place in close proximity to the sites of superoxide generation. Due to the negative charge of $O_2^{\cdot -}$ and its shorter half-life compared with NO (Vranova *et al.*, 2002), $ONOO^-$ production is more dependent on $O_2^{\cdot -}$ levels. Peroxynitrite can undergo homolytic cleavage to give HO· but its main biological function may be to react with tyrosine residues within proteins to form 3-nitrotyrosine. This occurs via addition of a nitro group in the ortho position of the aromatic ring in tyrosine (Kolbert *et al.*, 2017). The formation of nitrotyrosine represents an irreversible oxidative post-translational modification that is accepted as a biomarker of nitrosative stress. The incorporation of a nitro group into protein tyrosine can significantly change protein structure with consequent functional defects (e.g. enzyme inactivation). In plants, so far only loss of function by nitration has been reported (Begara-Morales *et al.*, 2013; Kolbert *et al.*, 2017). Nitration is a mechanism distinct from *S*-nitrosation where NO can covalently but reversibly oxidize the thiols groups of reduced reactive cysteine residues in proteins. *S*-Nitrosylation can activate or repress protein function (Gupta, 2011) and can be assessed using biotin-switch technology (BST). BST is currently the most commonly used method used for identification of nitrosylated proteins (Jaffrey and Snyder, 2001; Palmieri *et al.*, 2010) which is so well established that it will not be considered in this review. An excellent consideration of BST can be found in many papers; for example, Forrester *et al.* (2009).

Given the importance of NO, and its interaction in plant physiology, this review will critically consider current methods to measure NO. Measuring poses particular problems which will be discussed. In doing so, we will attempt to highlight the best practices so that the functions of NO can be assessed.

Assays for nitric oxide

NO quantification in tissues can be difficult due to the potential interference of various metabolites and its half-life, which can be extremely short, ranging from a few seconds to minutes. Therefore, it is necessary to use sensitive methods to detect NO. Further, as each method has advantages and disadvantages, it is recommended to use at least two methods for NO quantification (Gupta and Igamberdiev, 2013). There are several methods which are being used widely throughout plant and animal systems for the measurement of NO, such as fluorescent probes, the Griess reaction, EPR, chemiluminescence, oxyhaemoglobin (Hb-O_2) assay, laser photoacoustics, membrane inlet-mass spectrometry, and amperometric methods with NO-specific electrodes. These methods either measure free NO or oxidized products of NO such as NO_2^- or NO_3^-.

DAF or MNIP-Cu fluorescent probes

One frequently used method to detect NO is based on the fluorophore diaminofluorescein (DAF) in cell-permeable forms (DAF-2DA or DAF-FM DA) as developed by Kojima *et al.* (1998). The utility of these dyes have been demonstrated in various species, tissues types, and responses. Thus, DAF dyes have been used for live imaging of NO in cell suspensions (Planchet and Kaiser, 2006), tobacco leaves (Cvetkovska and Vanlerberghe, 2012), lateral root primordia of tomato (Correa-Aragunde *et al.*, 2004), and differentiating xylem of *Zinnia elegans* vascular bundles (Gabaldon *et al.*, 2005). Other examples include legumes, such as *Medicago truncatula* suspension cells and leaves (Kepczynski and Cembrowska-Lech, 2018), protoplasts of soybean roots treated with indole-3-acetic acid (IAA) (Hu *et al.*, 2005), and in vascular tissue (xylem), epidermal cells and root hairs of pea seedlings (Corpas *et al.*, 2006). DAF dyes have also been used to provide spatial information on NO generation patterns in response to various

stresses (Arita *et al.*, 2006; Planchet and Kaiser, 2006; Neill *et al.*, 2008; De Michele *et al.*, 2009; Mur *et al.*, 2011; Wany *et al.*, 2017, 2019; Vishwakarma *et al.*, 2018). Examples include tobacco epidermal (abaxial) sections treated with cryptogein, a fungal elicitor from *Phytophthora cryptogea* (Foissner *et al.*, 2000), or within developing *B. graminis* fungal sp. *hordei* appressoria (Prats *et al.*, 2008).

The reaction mechanism of DAF-2DA, is shown in Fig. 1(a). Once the DAF dyes enter the cell, ester bonds are hydrolysed by cellular esterases and DAF-2 is released. The resulting DAF-2 is *N*-nitrosated, forming the highly fluorescent triazolofluorescein (DAF-2T) which can be easily detected by fluorescence microscopy or fluorimetry. The advantage of DAF dyes is that they are highly sensitive (detection limit in the nanomolar range), non-invasive, readily commercially available, cost-effective, and easy to handle. It should be noted that DAF-2 does not directly react with NO but rather with N_2O_3 (Kojima *et al.*, 1998), hence DAF fluorescence depends on auto-oxidation of NO, which in turn is influenced by the cellular redox status (Planchet and Kaiser, 2006). However, NO_2^-, NO_3^-, $ONOO^-$, and H_2O_2 do not cause DAF-2DA to fluoresce (Kojima *et al.*, 1998). This specificity of DAF dyes was questioned when it was found that DAF-2 reacts with dehydroascorbic acid (DHA) and ascorbic acid to produce compounds that fluoresce within the range of DAF-2T. To overcome this, Ye *et al.* (2004), devised a method of assaying NO by freezing DAF-2 on dry ice. The NO present in the assay diffuses and reacts with frozen DAF-2, but the other less mobile compounds such as DHA would not be able to react (Zhang *et al.*, 2002). DAF-2DA was later replaced by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) that possesses greater photostability and sensitivity towards NO (~3 nM in comparison with 5 nM of DAF-2DA) (Kojima *et al.*, 1998). However, DAF-FM-DA has limited use, due to its pH sensitivity (Vitecek *et al.*, 2008), which means that it exhibits different pH values in plant cell compartments during different

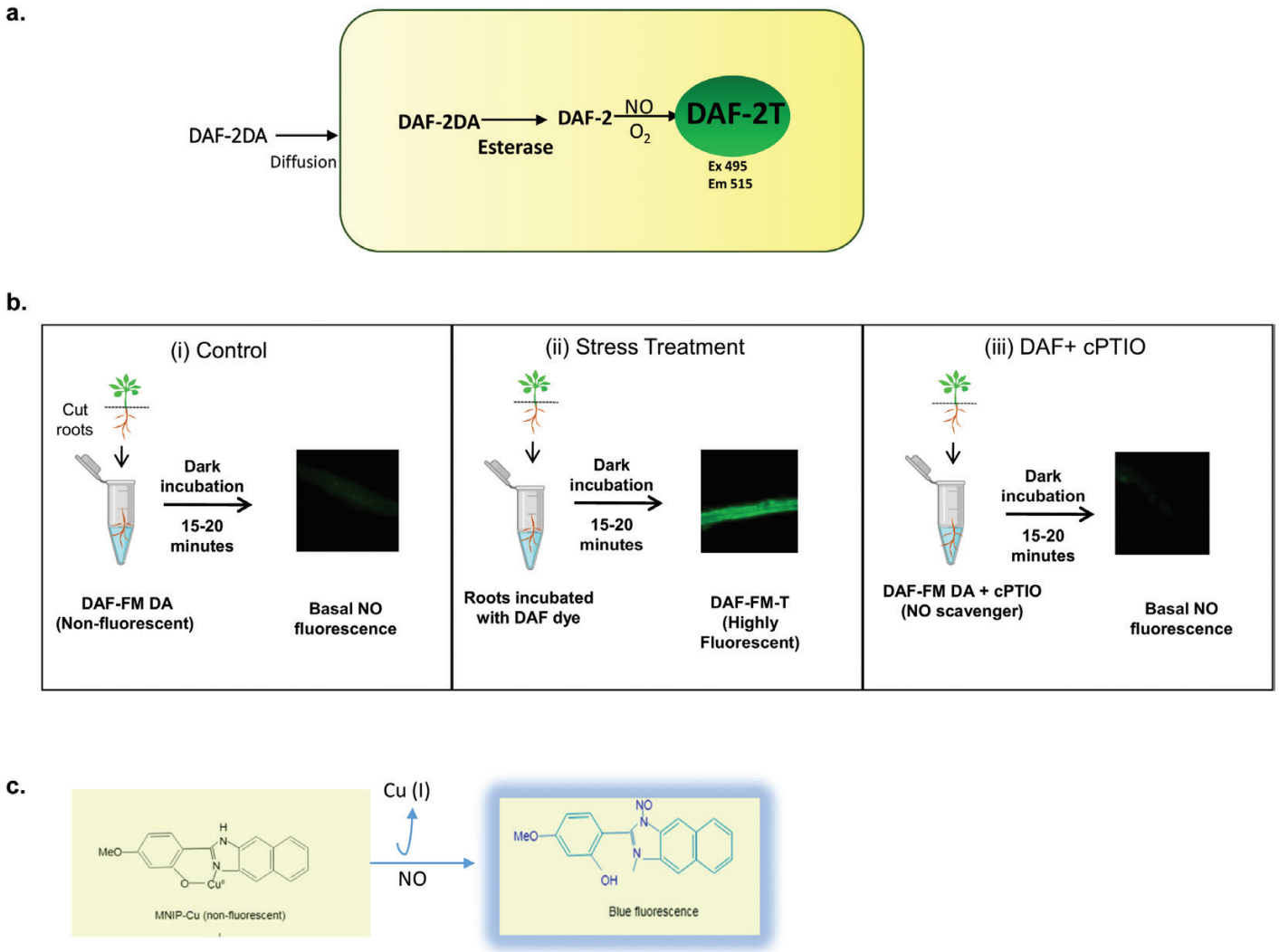

Fig. 1. *In vivo* NO fluorescence visualized by using DAF fluorescent dyes. (a) Reaction mechanisms of DAF-2DA. The diacetate groups of DAF dyes are removed by intracellular esterases, the diffused DAF-2 in the presence of NO (and O_2) forms highly fluorescent DAF-2T. (b) Suggested use of DAF dyes for measurement of NO under stress conditions. (i) Shows DAF fluorescence in roots of control seedlings incubated in DAF-FM-DA (10 μM in 50 mM HEPES buffer, pH 7.2) for 15 min in the dark. (ii) NO production under stress conditions; after incubation, roots shows intense DAF-FM fluorescence. (iii) Similarly, roots from stress-induced plants display basal NO fluorescence when cPTIO (an NO scavenger) is used along with DAF-FM under the same incubating conditions. (c) MNIP-Cu probe reacts directly with NO and gives blue fluorescence at 420 nm.

stresses, such as hypoxia, pathogen attack, and osmotic stress, which can lead to artefactual 'NO' measurements.

This method requires verification using the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). To unequivocally demonstrate that NO is being measured using the DAF dyes, the signal needs to be reduced through the application of cPTIO. This molecule reacts with NO to form the NO_2· radical and cPTI (Akaike and Maeda, 1996).

To aid investigators who may be new to the use of DAF dyes, we present a suggested approach in Fig. 1(b).

This is a fluorescence-based method which can be observed using confocal or fluorescence microscopy. MNIP-Cu is a cell-permeable fluorescent dye which forms a complex by direct reaction with NO and gives blue fluorescence (Fig. 1c). Its excitation and emission are in the ranges 330–385 nm and 420–500 nm, respectively. Dye is prepared by mixing MNIP with $CuSO_4$. This probe appears to be more specific for NO detection and it can be a better alternative to DAF dyes. MNIP-Cu has an extra advantage as it is sensitive under both anoxic and oxygen-rich conditions (Keisham *et al*., 2019). Yadav *et al*. (2013) demonstrated the auxin-induced NO detection in the hypocotyls of sunflower using MNIP-Cu. MNIP-Cu was also used for a localization study in whole seedlings, hypocotyl segments, stigma, and protoplasts of sunflower (*Helianthus annuus* L.) (Jain *et al*., 2016; Keisham *et al*., 2019).

Griess reaction

Plant researchers have used the Griess reagent to measure the nitrite level during low oxygen, seed germination, and plant–pathogen interactions (Doherty and Cohn, 2000; Liu *et al*., 2007; Vandelle and Delledonne, 2008; Vitecek *et al*., 2008; Wany *et al*., 2017; Vishwakarma *et al*., 2018). In addition, Griess reagent is also used in measurement of NR activity via change in nitrite concentration in a time course. In this method, NR activity is measured as nitrate to nitrite reduction coupled with NAD(P)H (Planchet *et al*., 2005; Wany *et al*., 2017).

This method was developed by a German chemist, Johann Peter Griess, in 1879. In this assay, nitrite is detected by reaction with sulfanilic acid and α-naphthylamine under acidic conditions to produce azo dye which is magenta in colour, which can be measured in a spectrophotometer at 540 nm. In subsequent developments of the assay, sulfanilic acid and α-naphthylamine were replaced by sulfanilamide and *N*-(1-napthyl) ethylenediamine (NED), respectively. The Griess reaction is actually an indirect measurement of NO. The available NO in the sample is oxidized to NO_2^- (as shown below in the reaction) and followed by reaction with Griess reagent which produces a magenta colour (Fig. 2a).

$$2NO+O_2 \rightarrow 2NO_2$$
$$NO+NO_2 \rightarrow N_2O_3$$
$$N_2O_3+H_2O \rightarrow 2NO_2^-+2H^+$$

The nitrite levels in unknown samples are calculated with a standard curve of nitrite (1–10 μM range). The advantage of this method is that it is quick, inexpensive, and can be used in any molecular biology laboratory. Further, a great advantage of this Griess reagent assay is that it can also be used for measurement of NO emitted from samples via the gas phase. Here, released NO is oxidized and reacts with sulfanilamide and NED to yield azo dye (Wany *et al*., 2017). This simple set-up is described in Fig. 2(b).

The exact methodology used in the Griess assay can vary between research groups. In some studies, sulfanilamide and NED (prepared in acidic medium: HCl/H_3PO_4) are added together to the nitrite sample (Planchet and Kaiser, 2006; García-Robledo *et al*., 2014; Vishwakarma *et al*., 2018). Alternatively, sulfanilamide is first allowed to react with nitrite, followed by NED after a 10 min incubation (Wany *et al*., 2017).

EPR spin trap

EPR spectroscopy is a reliable method to detect the short-lived NO radical. EPR is based on detection of unpaired electrons in magnetic fields within a microwave cavity which display a 'resonance' between parallel and an antiparallel electron spin orientations (Kleschyov *et al*., 2007) (Fig. 3). EPR is aided by the use of a chemical 'spin trap' that produces persistent paramagnetic adducts which are stable for minutes to hours to allow their detection (Steffen-Heins and Steffens, 2015). Use of a spin trap is essential for NO detection, given its very low concentration and short half-life. The principle of a spin trap is as follows:

$$R^{\star}+ST \rightarrow R\text{-}SA$$

where $R^{\star}$ is the radical intermediate, ST is a spin trap, and R-SA is the radical spin trap adduct that is detected by EPR spectroscopy.

EPR sensitivity in detecting NO and other radicals depends on various factors such as the spin trap used, transient radical concentration, the reaction kinetics of the radicals to form adducts, and their stability (Steffen-Heins and Steffens, 2015). If used correctly, this method can detect pmoles of NO (Weaver *et al*., 2005).

NO spin-trapping agents can be endogenous in origin such as Hb or other metalloproteins, or exogenous compounds such as diethyldithiocarbamate (DETC) and *N*-methyl-D-glucaminedithiocarbamate (MGD). Hbs represent an attractive option for use as an NO spin trap agent. They can react with NO very rapidly in both oxy- and deoxy-Hb forms. Met-Hb can also be used to detect NO by this method. The interaction of NO and Hb leads to formation of paramagnetic Hb/NO derivatives which can be detected by EPR (Kosaka, 1999).

Use of iron-dithiocarbamates in EPR exploits the high affinity of NO for iron. Iron-dithiocarbamate ST [$Fe(S_2CN\text{-}R R')_2$] side groups include methyl-, ethyl-, glucamine, sarcosine, or amino acids. This R side group variation can be useful for targeting NO (Kleschyov *et al*., 2007). 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxy-3-oxide (PTIO) or cPTIO (a class of imidazolineoxy-*N*-oxides) can be used as spin traps, and these compounds react with rate constants of the order of 10^4 mol^{-1} s^{-1} with concomitant generation of NO_2^-/NO_3^-. This method has some limitation as the cPTIO (NNO) produced in the reaction is degraded within a few minutes.

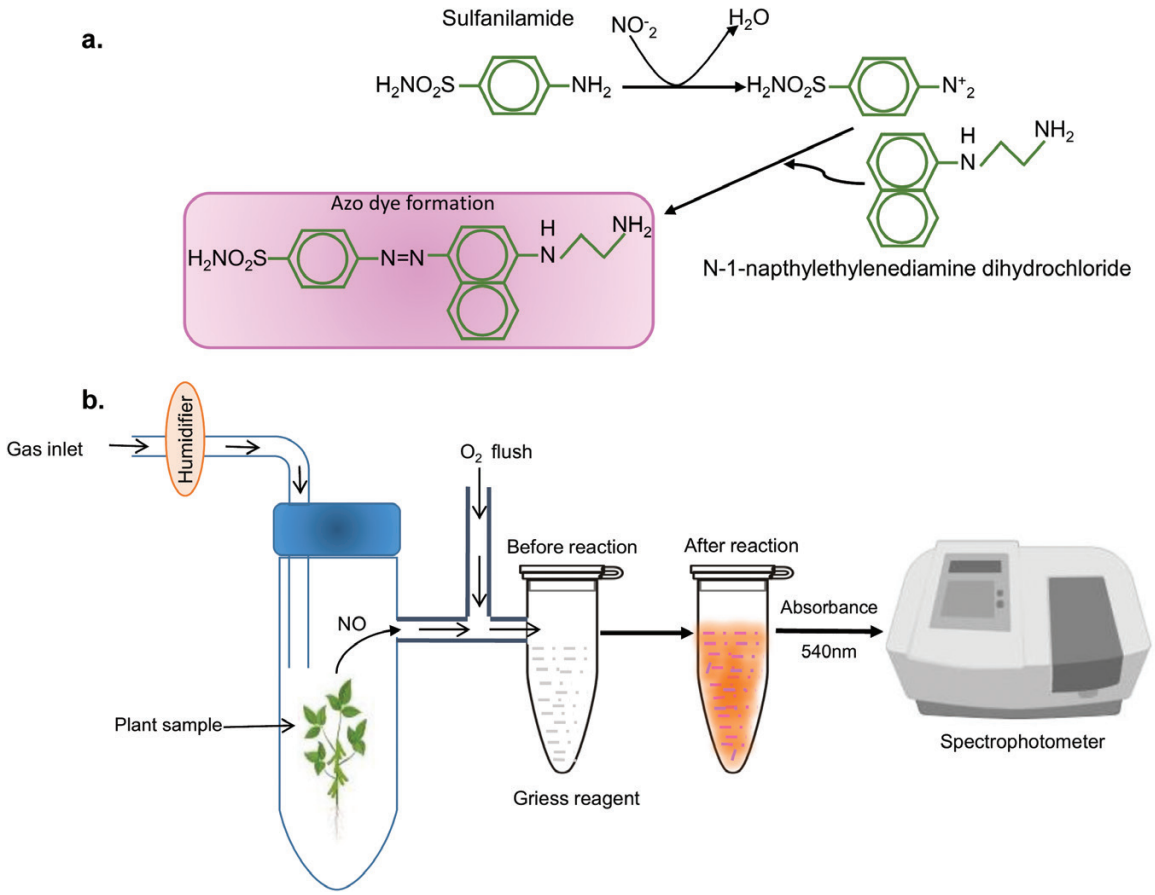

Fig. 2. Detection of gaseous nitric oxide in plants through the Griess assay. (a) Reaction mechanism which shows that NO is first oxidized to nitrite and reacts with sulfanilamide to give a diazonium salt. The diazonium salt then reacts with *N* -(1-naphthyl) ethylenediamine (NED) and forms a magenta coloured azo compound showing absorbance at a maximum of 540 nm. (b) Set-up for measurement of NO using the Gas Phase Griess reagent.

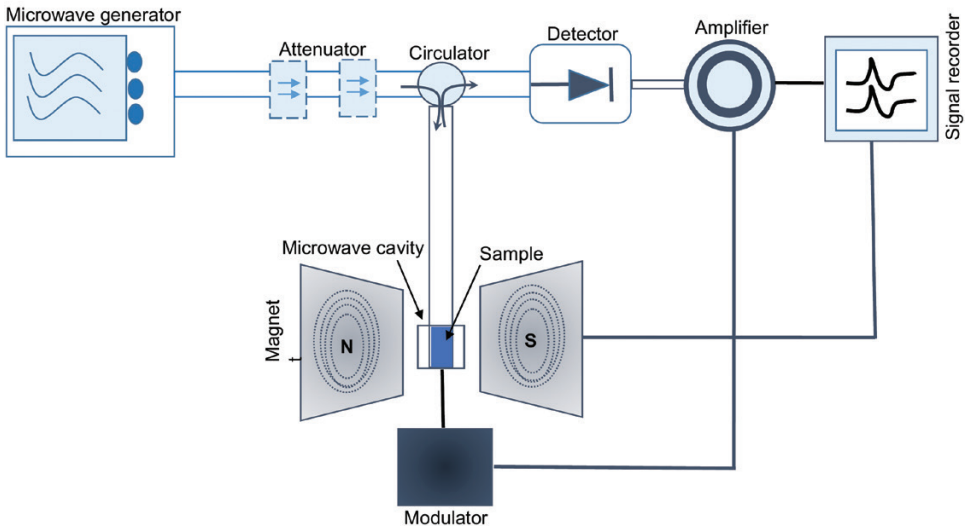

Fig. 3. A simple illustration of EPR spectroscopy employing magnetic field modulation. The microwave bridge typically operates at 9.5 GHz, with modulation and demodulation circuits at 100 kHz. Magnetic scanning is done very slowly (i.e. 10–100 mHz).

INO, a compound, generated by a reaction between cPTIO and NO, has not been detected in Arabidopsis cell cultures (D'Alessandro *et al.*, 2013).

Example studies which used EPR to include the measurement of NO in wounded *Arabidopsis thaliana* leaves using [Fe(II)$(DETC)_2$], linked NO to jasmonic acid signalling (Huang *et al.*,

2004), metal-induced programmed cell death (De Michele *et al.*, 2009), innate immunity (Zeidler *et al.*, 2004), and its effect on chloroplastic lipids and protein (Jasid *et al.*, 2006). Bright *et al.* (2009) reported that all hydrated pollen produce NO, which might have a possible link to allergic responses such as rhinitis (hay fever) in humans.

The EPR approach can also be elaborated through the use of ^{14}N and ^{15}N labels in experiments so that both NO and its enzymatic source can be measured (Maia and Moura, 2016). EPR spectroscopy has been used to show the NO_2^--dependent NO synthesis upon the *Pseudomonas syringae*-elicited hypersensitive response, in NR-deficient *A. thaliana* (Modoloa *et al.*, 2006). In this method, the sample extract was incubated with labelled $^{15}NO_2^-$, $^{15}NO_3^-$, or l-($^{15}N_2$-guanidineimino) arginine and the spin trap $(MGD)_2Fe(II)$. The NO production was quantified by measuring the levels of labelled nitrosyl complex $(MGD)_2Fe(II)^{15}NO$.

In considering whether to use EPR spectroscopy, its advantages and disadvantages need to be weighed up. EPR advantages include direct assay, high specificity due to use of NO spin traps, no interference with NO_2^- and NO_3^-, and compatibility for nitrogen isotope substitution. However, its disadvantages are considerable, particularly its cost. It is also semi-quantitative and requires special expertise to analyse the derived data. In addition, the method may generate and artificially detect the NO from HNO, nitrite, and *S*-nitrosothiols (Bellavia *et al.*, 2015; Csonka *et al.*, 2015).

Chemiluminescence method

Of all of the various methods developed for detecting NO, chemiluminescence is one of the most robust and widely used. This method is based on the reaction of NO with ozone (O_3), which emits a quantum of light. The light produced is directly proportional to the amount of NO produced in the biological system in a given time (Gupta *et al.*, 2005; Planchet *et al.*, 2005).

The constituent reactions are the reaction of NO with O_3 to produce nitrogen dioxide in the excited state,

$$NO+O_3 \rightarrow NO_2^{\star}+O_2$$

In the second step, $NO_2^{\star}$ returns to its ground state which leads to emission of photons ($h\nu$),

$$NO_2^{\star} \rightarrow NO_2+h\nu$$

The photons emitted can be counted in a photomultiplier tube (Gupta *et al.*, 2005; Planchet *et al.*, 2005).

The chemiluminescent assay is exploited with often commercially available equipment with different parts (Fig. 4a). The first part is the sample chamber whose dimensions can be changed depending on sample number and size. For instance, a chamber can be designed to measure NO from a whole plant or it can be a very small (2–5 ml) beaker that can be used to assess cells and organelles such as mitochondria. Secondly, a constant source of gas flow (air or N_2) with an adjusted flow rate (1 or 1.5 l min^{-1}) is maintained inside the

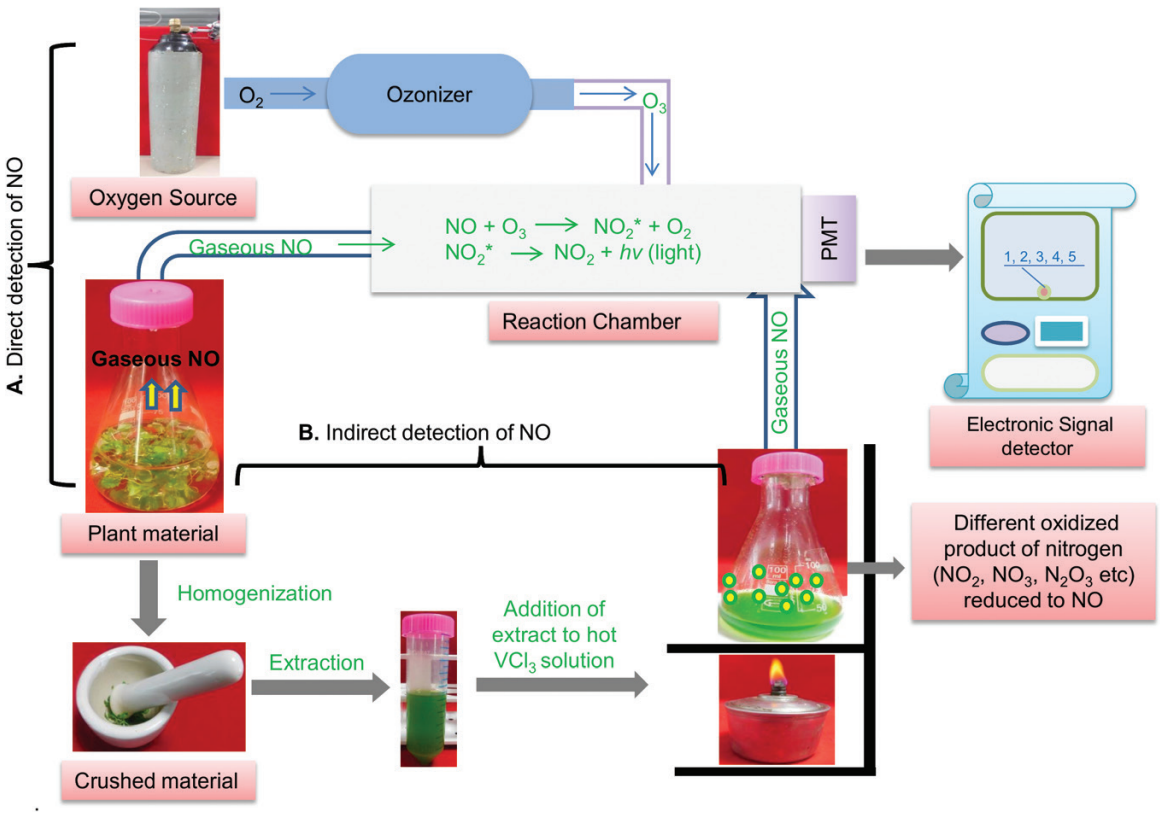

Fig. 4. Chemiluminescence detection of nitric oxide from plant samples. (a) Direct detection of chemiluminescence by passing released NO from plant material to the reaction chamber containing ozone. NO reacts with ozone, leading to formation of NO_2^* which then jumps to the ground state emitting light which is proportional to the amount of NO produced. (b) Indirect detection of chemiluminescence by injecting plant extract into hot acidic VCl_3 where released NO passes into the reaction chamber containing ozone and then signals are detected.

chamber by flow controllers. The chamber is connected to the chemiluminescence detector which is also linked to an ozone generator. The NO signal can be calibrated through the use of NO-free air.

The chemiluminescence method is highly sensitive, with an NO detection limit down to a pmole range (Planchet *et al.*, 2005). There are many example studies where the chemiluminescence method was used to measure, from cell suspensions, root and leaf tissues, organelles of various species of plants (reviewed in Wany *et al.*, 2016). This method has a significant advantage over other methods as it allows NO measurements from both gaseous and aqueous samples. If the biological sample is aqueous, NO can be driven to gas phase by bubbling the solution with an inert gas or shaking the sample (Gupta *et al.*, 2005; Planchet *et al.*, 2005).

A potential disadvantage is that chemiluminiscence only measures gaseous NO and no other forms of nitrogen or reactive nitrogen intermediates (Planchet and Kaiser, 2006). Thus, the considerable levels of NO that are oxidized in living tissues cannot be easily detected by using this method (Table 1). However, by using an indirect chemiluminiscence method, the oxidized forms of NO such as NO_2^- and NO_3^- can be reduced back to NO and therefore detected. For this, sample extracts must be injected into heated (95 °C) acidic vanadium chloride (VCl_3); subsequently, it generates chemiluminescence, the NO signal, which is recorded in the signal detector sensitive enough to trap parts per billion (ppb) amounts of NO (Fig. 4b) (Rumer *et al.*, 2009).

Oxyhaemoglobin assay

During various physiochemical reactions occurring inside the cell, NO can interact with the Hb moiety in several ways such as occupying Hb's vacant haems in a cooperative manner such as nitrosylation of Hb thiols or by reacting with superoxide produced during biochemical reactions (Gow *et al.*, 1999). This oxidative reaction between NO and HbO_2 generates Met-Hb and nitrate, which results in a very simple and reliable method for estimating NO produced in living systems, known as the oxyhaemoglobin assay (Feelisch *et al.*, 1996). This quantitative assay for NO estimation using a spectrophotometer was first described by Feelisch and Noack (1987). The reaction mechanism involves the shift in absorbance from 415 nm which belongs to HbO_2 to 401 nm due to formation of Met-Hb (Fig. 5) (Larfars and Gyllenhammar, 1995). The available NO can be quantified by measuring the difference in absorbance at 401 nm and 421 nm (Murphy and Noack, 1994). The HbO_2 assay can detect NO in the range of 1.3–2.8 nM in a short time, so it is a very fast and sensitive method (Murphy and Noack, 1994). HbO_2 assay has been used widely in many plant studies related to NO signalling. Orozco-Cardenas and Ryan (2002) reported that NO negatively modulates wound signalling in tomato plants. They used excised leaves of wounded or non-wounded tomato and homogenized them in 1 ml of ice-chilled buffer containing 0.1 M sodium acetate, 1 M NaCl, and 1% (w/v) ascorbic acid, pH 6.0. Supernatant collected after centrifugation at 10 000 *g* for 20 min at 4 °C, passed through 0.8×4 cm columns in 1×8 resin, was used for spectrophotometric analysis by measuring the conversion of HbO_2 to Met-Hb. The reaction mechanism is as follows:

$$HbO_2 + NO \rightarrow MetHb + NO_3^-$$

A fresh calibration is required before each experiment. Calibration is performed by oxidation of HbO_2 with potassium ferricyanide, with an NO donor (SNP, SNAP, and GSNO) that generates a known concentration of NO (Hetrick and Schoenfisch, 2009). Ghafourifar *et al.* (2005) used this method for the measurement of mitochondrial NOS activity. However, this method is not in regular use for several reasons. The first reason is that ROS produced inside the cell can also oxidize HbO_2 that can give false readings. This can be prevented by addition of superoxide dismutase and catalase during measurement (Ma *et al.*, 2016). Secondly, variation in pH, which is a common phenomenon occurring inside a plant cell, can affect the sensitivity of this assay (Mur *et al.*, 2011). In addition, temperature change, and the presence of haem-containing proteins can also interfere with the assay as these parameters influence the absorbance spectrum of Met-Hb (Noack *et al.*, 1992).

Mass spectrometry

Conrath *et al.* (2004) demonstrated the use of membrane inlet mass spectrometry (MIMS) to detect NO by a non-invasive isotope pairing technique. MIMS can quantify NO in aqueous solution, but the RIMS (restriction capillary inlet mass spectrometry) approach can measure NO from the gas phase. The MIMS set-up comprises a semi-permeable membrane as an inlet which is connected to a mass spectrometer (Fig. 6a). Samples such as cell suspensions should be stirred and passed through a Teflon membrane (50 μm) that is permeable to NO, following which it is subsequently analysed by MS. In RIMS, NO passes directly through the samples, such as leaves, to the mass spectrometer (Fig. 6b).

The advantage of this method is that it has the ability to distinguish different isotopic forms of ^{15}N-labelled nitrate, nitrite, arginine, and hydroxylamines. N_2 isotopes can also be calculated; thus quantification can be achieved by this method. A combination of MIMS/RIMS can allow the fast, specific, and simultaneous detection of NO, O_2, and CO_2 from the same sample (Conrath *et al.*, 2004). Indeed, this was demonstrated following the addition of nitrate to mouse macrophages, plant tissue cultures (tobacco, parsley, and soybean), tobacco leaves, whole Arabidopsis plants, green algae, fungi such as *Pythium* sp., *Botrytis* sp., and *Fusarium* sp., and some species of cyanobacteria (Conrath *et al.*, 2004). In all these assays, oxygen concentration was maintained at <1%. However, this technique did not detect NO release when a plant or its leaves is sprayed with equimolar concentrations of phosphate or even water. The technique can be extended to detect *S*-nitrosothiols coupled with LC or GC (Palmieri *et al.*, 2010).

This superficially attractive method is, however, not often used, most probably due to the expensive equipment required (Table 1).

Table 1. *Advantages and disadvantages of NO methods*

No	Method	Advantages	Disadvantages
1	DAF-FM, DAF-FM-DA, and DAF-2DA	(i) Commercially available in market (ii) Highly sensitive; measures NO levels at nanomolar concentrations (iii) Allows cellular localization studies	(i) pH sensitive (ii) Requires oxygen (iii) DAF-2 does not directly react with NO (iv) The uptake of DAF-2DA varies with tissue type and organelle (v) Forms an N_2O_3 intermediate in the reaction (vi) Ascorbic and dehydroascorbic acid can react with DAF (vii) Accurate quantitative measurement is not possible
	MNIP-Cu fluorescent probes	(i) Cheaper than DAF (ii) Reacts directly with NO (iii) MNIP-Cu probe can measure NO at anoxic, hypoxic, and normoxic conditions	(i) Precise quantitative measurement is not possible
2	Griess reaction	(i) Rapid and inexpensive (ii) Possible to measure NO in the gas phase	(i) Poor sensitivity compared with other methods (ii) Quantification can be difficult (iii) Not applicable to cellular localization studies
3	EPR spin trap	(i) NO can be measured down to picomolar concentrations (ii) Used properly, EPR spectroscopy is highly specific to NO	(i) A semi-quantitative method requiring complex methodology to acquire and analyse the results (ii) Online measurement of NO is difficult (iii) Spin traps can be expensive (iv) Equipment is expensive and requires specialist expertise to be used properly (v) Not applicable to cellular localization studies (vi). It cannot measure NO emitted into the gas phase
4	Chemiluminescence	(i) Measures NO in gas phase (ii) NO measurement in the ppb range is possible (iii) Highly reliable (iv) Can be used to measure NO from *in vitro* enzymatic reactions, tissues, and organelles	(i) Difficult to measure oxidized forms of NO (ii) Equipment is expensive and requires specialist expertise to be used properly (iii) Not applicable to cellular localization studies
5	Oxyhaemoglobin assay	(i) A sensitive method, measures NO levels in the range of 1.3–2.8 nM (ii) Specialized equipment is not required	(i) Intracellular ROS can interfere with the formation of methaemoglobin complex leading to false-positive results. (ii) Rapid pH and temperature changes in the cellular environment can affect the method's sensitivity (iii) Several haem-containing proteins fall within the range of the same absorbance (iv) Not applicable to cellular localization studies
6	Mass spectroscopy	(i) Quantification of NO can be done efficiently by using ^{15}N isotopes (ii) The combination MIMS/RIMS allows the simultaneous detection of NO, O_2, and CO_2	(i) Equipment is expensive and requires specialist expertise to be used properly (i) Not applicable to cellular localization studies (iii) Expensive and expertise is needed
7	Quantum cascade laser	(i) A very sensitive method, measuring NO down to ppb range (ii) Allows online, *in vivo* NO production to be determined	(i) Equipment is expensive and requires specialist expertise to be used properly (ii) Requires interdisciplinary approaches to analyse the results (iii) Can only measure NO in the gas phase (iv) Localization is not possible

Quantum cascade laser

Quantum cascade laser (QCL) is one of the most advanced methods for NO detection. A QCL-based detector works at ambient temperature and is highly applicable for gas sensing due to its high sensitivity (parts per billion), compact size, excellent spectral quality, and low power requirements. NO measurements using QCLs have recently been reviewed by us (Montilla-Bascon *et al*., 2018), so are only briefly considered here.

For NO detection, a QCL emitting light at 5.3 μm (1850 cm^{-1}) is required. There are two different lasers currently being used, namely AllnAs/GalnAs- and GaAs/AlGaAs-based materials. A gas line from the sample being analysed is passed into a gas cell which has mirrors at both ends. This allows multiple reflections of the laser light inside the cell so that a cell that could be 30 cm long has an effective path length of 76 m. This explains the term 'multi-pass gas cell' in QCL detection. The NO concentration is determined from comparison with a gas mixture of known NO concentrations in nitrogen. For long-term reproducibility of sample measurement, the optical set-up is placed in a temperature controlled box.

One advantage of this QCL-based NO detector over chemiluminescence is the possibility of long-term measurements due to a low ventilation rate over the sample (usually 1–1.5 l h^{-1}) so that the sample can be affected by dehydration. However, the disadvantages are significant as the QCL approach requires considerable specialist equipment and expertise. Significantly, most NO

measurement studies using QCL have been based on collaborations between biologists and physicists. An example of this is a comparison of the sensitivity of the QCL and chemiluminescence approach in tomato plants infected with *Botrytis cinerea*. Data obtained from both methods showed identical NO detection levels (Mur *et al.*, 2011). There is the potential for QCL miniaturization to make it more amenable for general laboratory use, but currently interdisciplinary collaboration remains the best means to access and exploit this power approach to NO detection.

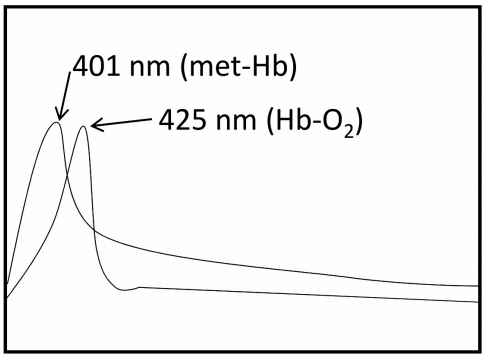

Fig. 5. A simple diagram shows the absorbance peak of Met-Hb and HbO_2. The reaction of NO with Met-Hb causes a shift in the peak from 401 nm to 411 nm which indicates the oxidation of Met-Hb into HbO_2.

NO electrodes

Measurement of NO by electrodes is one of the cheapest and alternative ways to measure NO. In a typical NO electrode, the Teflon/platinum serves as the main electrode and Ag/$AgCl_2$ as the reference electrode (Besson-Bard *et al.*, 2008a). The main and reference electrodes are enclosed in a glass or steel tube filled with a solution of 0 mM NaCl/0.3 nM HCl. The open end is covered with an NO-permeable membrane. In this method, NO can be detected based on its oxidation at +0.8 V to +0.9 V in comparison with the reference electrode (Shibuki, 1990). These can be constructed in a less expensive way (Besson-Bard *et al.*, 2008a), cell death in tobacco BY2 cells (Ma *et al.*, 2010), and cryptogein-induced cell death (Besson-Bard *et al.*, 2008a). The disadvantage is that NO emitted into the gas stream cannot be measured by using this method.

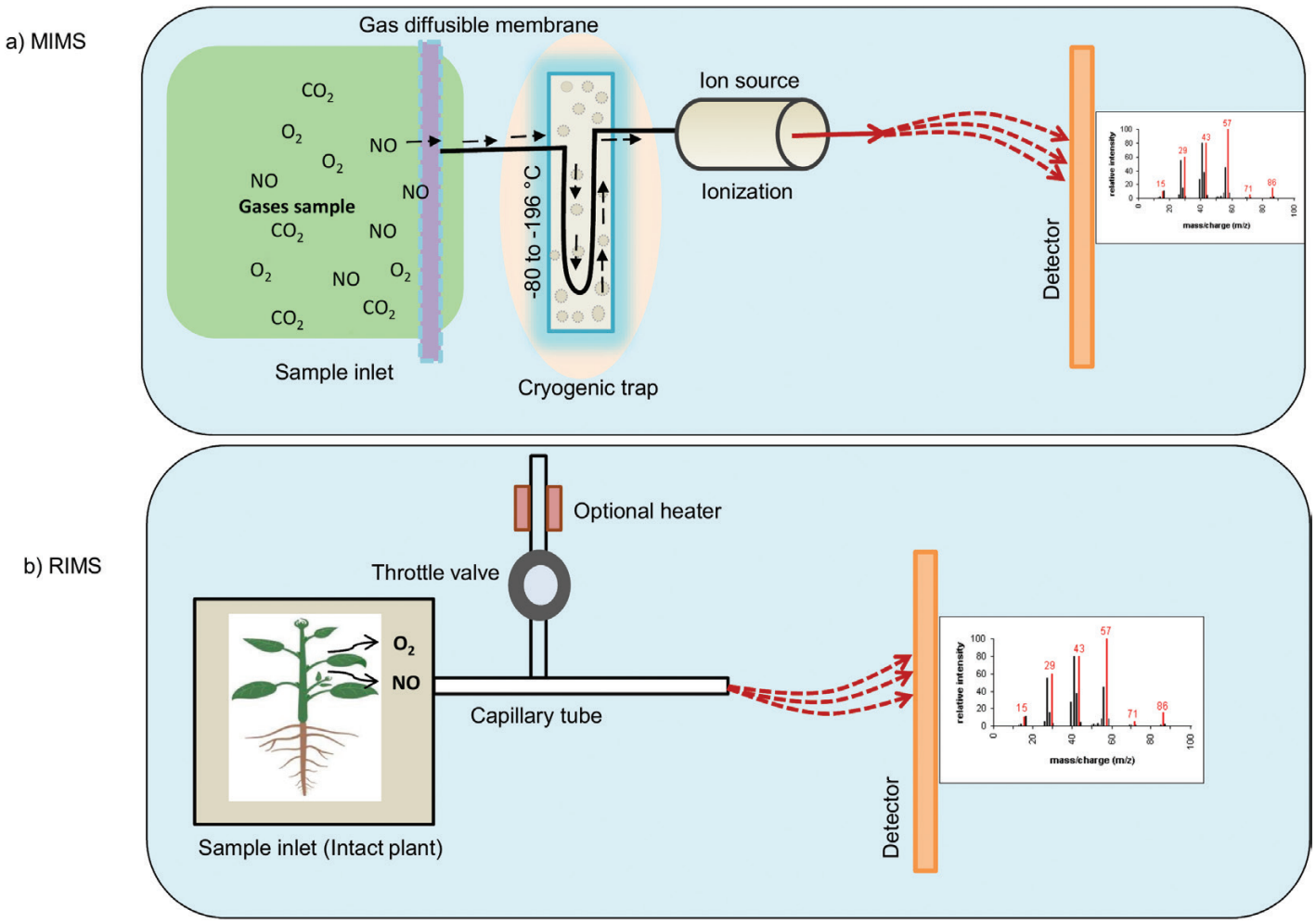

Fig. 6. Schematic view of the experimental set-up of MIMS- and RIMS-based NO measurement. (a) In MIMS, a cell suspension in an 8–10 ml reaction chamber is circulated over a thin Teflon membrane by a magnetic stirrer. The dissolved gases diffuse through the membrane and pass through the ionization chamber of the mass spectrometer. (b) In RIMS, the sample can be a leaf of a small plant or root tissue cells which fit into the leaf cuvette. The produced gases pass through the capillary tube before entering the ionization chamber of the mass spectrometer. A throttle valve connected to a capillary tube regulates the flow of gases to the ionization chamber.

Conclusions

In conclusion, in this review we have considered various means of NO mneasurement. The list is not exhaustive, but reflects the experiences of the authors. In each case, we recommend care being used to confirm that the signals obtained truly reflect the radical molecule being assessed, ideally through the use of scavengers, inhibitors, mutants, or overexpressing lines. If possible, more than one assay should be used to corroborate the results, although for practical reasons this may be not possible.

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