Current approaches to measure nitric oxide in plants

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Highlight: This review summarizes current approaches to detect nitric oxide and discuss advantages and disadvantages of each method

Abstract

Nitric oxide (NO), is now established as an important signalling molecule in plants where they influence growth, development and responses to stress. Despite of extensive research, the most appropriate methods to measure and localise these signalling radicals are debated and still needs investigation. Many confounding factors such as 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 the sensitive and specific methods in order to understand the contribution of each signalling molecule to various biological processes. In this review, we will provide current state knowledge on NO measurement in plant samples, via various methods. We will also discuss advantages, limitations and wider applications of each method.

Key words:

Nitric oxide, redox, mitochondria, chemiluminiscence, hemoglobin, Quantum cascade laser

Introduction

The gaseous free radical nitric oxide (NO) emerged as an important signaling 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; Domingos *et al.*, 2015; Fancy *et al.*, 2017; Wilson *et al.*, 2008; Yu *et al.*, 2014).

In case of animals, NO acts as a potent endogenous vasodilator and plays important roles in inflammation, inhibition of platelet aggregation and regulation of immune responses, cGMP signalling, protection during ischemia and reperfusion injury (Phillips *et al.*, 2009; Schmidt and Walter, 1994). In animals, the majority of NO is produced by a family of nitric oxide synthases (NOS). NO catalyses deamination of arginine to citrulline. Three NOS enzymes are characterized in animal systems i.e., 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 co-factors such as NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4), calcium/calmodulin etc. In addition to NOS, mitochondria of animal system are also known to produce NO via nitrite dependent pathway (Shiva, 2010).

Comparison of NO signalling in animals and plants, revealed several commonalities (Wendehenne *et al.*, 2001). Plant NO signalling also operates via S-nitrosylation process and also via activation of calcium, protein kinase cGMP signalling and ROS (Gaupels *et al.*, 2011; Wendehenne *et al.*, 2001). Despite of several attempts to identify NOS, the clear picture on its existence in land plants has not been achieved 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 is mainly based on two methods, one is on 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 (N6-(1-Iminoethyl)-L-lysine) , L-NAME (NG-Nitro-L-arginine methyl ester), L-NMMA (NG-Monomethyl-L-arginine) and second is Arginine/Citrulline assay. The latter is recently questioned because of argininosuccinate formed as a side reaction synthesized from Arg and fumarate by argininosuccinate lyase can give false positive results (Tischner *et al.*, 2007). Recent evidence suggests that few algal species such as *Ostreococcus tauri* possesses 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 (Planchet *et al.*, 2005; Rockel *et al.*, 2002). 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 only 10% NR activity of 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 AOX (Gupta *et al.*, 2018). In addition, NO may be also 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 operates 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 characterised and needs further investigation (Rumer *et al.*, 2009; Tun *et al.*, 2006).

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 to NO (Vranova *et al.*, 2002), $ONOO^-$ production is more dependent on $O_2^{-\cdot}$ levels. Peroxynitrite can undergo homolytic cleavage to give $HO^\cdot$ but its main biological function may be to react with tyrosine residues within proteins to form 3-nitrotyrosine. This occurs via addition of nitro group in the ortho position of the aromatic ring in tyrosine (Tyr) (Kolbert *et al.*, 2017). The formation of nitrotyrosine represents an irreversible oxidative posttranslational modification that is accepted as a biomarker of nitrosative stress. The incorporation of 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 distinct mechanism to S-nitrosation where NO can covalently but reversibly oxidise 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 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 few seconds to minutes. Therefore, it is necessary to use the 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 the plant and animal systems for the measurement of NO such as fluorescent probes, Griess reaction, electron paramagnetic resonance,

chemiluminescence, oxyhemoglobin assay, laser-photoacoustics, Membrane Inlet-mass spectrometry, amperometric methods with NO-specific electrodes. These methods either measure free NO or oxidised 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), in lateral root primordia of tomato (Correa-Aragunde *et al.*, 2004), in 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 IAA (Hu *et al.*, 2005), in vascular tissue (xylem) and epidermal cells and in 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; De Michele *et al.*, 2009; Mur *et al.*, 2011; Neill *et al.*, 2008; Planchet and Kaiser, 2006; Vishwakarma *et al.*, 2018; Wany and Gupta, 2018; Wany *et al.*, 2017). Examples include, the 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, DAF-FM DA and DAF-FM is shown in Fig. 1a. Once the DAF dyes enter the cell, ester bonds are hydrolyzed 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 microscope or fluorimeter. The advantage of DAF dyes is that they are highly sensitive (detection limit in 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 with N_2O_3 (Kojima *et al.*, 1998), hence DAF fluorescence depends on auto-oxidation of NO, which in turn is influenced by cellular redox status (Planchet and Kaiser, 2006). However, NO_2^-, NO_3^-, and $ONOO^-$, 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, DAF2 reacts with dehydroascorbic acid (DHA) and ascorbic acid (AA) 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 react with frozen DAF-2, but the other, less mobile compounds such as DHA would not be able to react (Zhang *et al.*, 2002). DAF-2-DA was later replaced by 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM-DA) that possesses greater photo-stability and sensitivity towards NO (~3 nM in comparison to 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 they exhibit

different pH values in plants cell compartments during different stresses; such as hypoxia, pathogen attack, osmotic stress, can lead to artifactual "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 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 which may be new to the use of DAF dyes; we present an suggested approach in Fig. 1b.

This is a fluorescence-based method which can be observed using confocal or fluorescence microscope. MNIP-Cu is a cell permeable fluorescent dye which forms a complex by direct reaction with NO and gives a blue fluorescence. Its excitation and emission are 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 localization study in whole seedlings, hypocotyls segments, stigma and protoplasts of sunflower (*Helianthus annuus* L.) (Jain *et al.*, 2016; Keisham *et al.,* 2019).

Griess reaction

The 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; Vishwakarma *et al.*, 2018; Vitecek *et al.*, 2008; Wany *et al.*, 2017). In addition, Griess reagent is also used in measurement of NR activity via change in nitrite concentration in 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 condition to produce azo dye which is magenta in color which can be measured in 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 color (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 to 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 be also used for measurement of NO emitted from samples via gas phase. Here, released NO is oxidized and reacts with sulphanilamide and NED to yield azo dye (Wany *et al.*, 2017). This simple set up is described in Fig. 2b.

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

EPR spin trap

Electron paramagnetic resonance (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 a 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 its 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 spin trap is as follows:

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

Where R* is radical intermediate, ST is a spin trap and R-SA 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 pmols of NO (Weaver *et al.*, 2005).

NO spin-trapping agents can be endogenous in origin such as like haemoglobin (Hb) or other metalloproteins, or exogenous compounds such as diethyldithiocarbamate (DETC) and N-methyl-D-glucaminedithiocarbamate (MGD). Hemoglobins represent an attractive option for use as a NO spin trap agent. They can react with NO very rapidly in both oxy- and deoxy-hemoglobins forms. Met-Hemoglobin also can 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-dithiocarbamates 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 carboxymethoxy-PTIO (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 few

minutes. INO, a compound, generated by in 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)$(DETC)_2$], linking NO to jasmonic acid signaling (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 proteins in *Arabidopsis thaliana* (Jasid *et al.,* 2006). Bright *et al.*, (2009) reported that all hydrated pollen produce the NO which might have possible link in allergic responses e.g. 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 *Pseudomonas syringae* elicited hypersensitive response, in a NR deficient *Arabidopsis thaliana* (Modolo *et al.*, 2006). In this method, the sample extracts was incubated with labelled $^{15}NO_2^-$,$^{15}NO_3^-$ or L-($^{15}N_2$-guanidineimino) arginine and the spin trap $(MGD)_2$Fe(II). The NO production was quantified by measuring the levels of labelled nitrosyl complex $(MGD)_2$Fe(II)^{15}NO.

In considering whether to use EPR spectroscopy, its advantages and disadvantages need to be weighed. 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 expense. 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 robust and widely used method. 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,

$$NO + O_3 \rightarrow NO_{2^*} + O_2$$

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

$$NO_{2^*} \rightarrow NO_2 + h\nu$$

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 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 min^{-1}) is maintained inside the 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 a NO detection limit down to a pmol 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 significant advantage over other methods as it allows the 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, NO signal, which is recorded in the signal detector sensitive enough to trap parts per billion (ppb) amounts of NO (Rumer et al 2009; Fig. 4b).

Oxyhemoglobin assay

During various physiochemical reactions occurring inside the cell, NO can interact with the hemoglobin moiety in several ways such as occupying hemoglobin's vacant hemes in a cooperative manner such as nitrosylation of hemoglobin thiols or by reacting with superoxide produced during biochemical reactions (Gow *et al.,* 1999). This oxidative reaction between NO and oxyhemoglobin (oxyHb) generates methemoglobin and nitrate, which results in a very simple and reliable method for estimating NO produced in living systems, known as oxyhemoglobin assay (Feelisch *et al.*, 1996). This quantitative assay for NO estimation using spectrophotometer was first described by Feelisch and Noack, (1987). The reaction mechanism involves the shift in absorbance from 415 nm which belongs to oxyhemoglobin to 401 nm due to formation of methemoglobin (Fig 5) (Larfars and Gyllenhammar, 1995). The available NO can be quantified by measuring difference in absorbance at 401 and 421 nm (Murphy and Noack, 1994). Oxyhemoglobin assay can detect NO in the range of 1.3-2.8 nM with in short time, so it is very fast and sensitive method (Murphy and Noack, 1994). Oxyhemoglobin assay has been used widely in many plant studies related to NO signaling. Orozco-Cardenas and Ryan, (2002) reported that NO negatively modulates wound signaling in tomato plants. They used excised leaves of wounded or non- wounded tomato and homogenized 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 X 8 resin, was used for

spectrophotometric analysis by measuring the conversion of oxyhemoglobin to methemoglobin. The reaction mechanism is as follows:

$$HbO_2 + NO \rightarrow \text{met-Hb} + NO_3^-$$

A fresh calibration is required before each experiment. Calibration is performed by oxidation of HbO_2 with potassium ferricyanide, with a 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 because of several reasons. The first reason is that ROS produced inside the cell can also oxidise 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 plant cell can affect the sensitivity of this assay (Mur *et al.*, 2011). In addition, temperature change, and presence of heme-containing proteins can also interfere with the assay as these parameters influence the absorbance spectrum of MetHb (Noack *et al.*, 1992).

Mass spectrometry

Conrath *et al.*, (2004) demonstrated the use of membrane inlet mass spectrometry (MIMS) to detect NO by non-invasive isotope pairing technique, MIMS can quantifies NO in aqueous solution but in the RIMS (restriction capillary inlet mass spectrometry) approach can measure NO from the gas phase. The MIMS setup

comprises of a semipermeable 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 mass spectrometry. In RIMS, NO passes directly through the samples, such as leaves, to the mass spectrometer (Fig. 6b).

The advantage of this method is, it has 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, soybean), tobacco leaves, whole Arabidopsis plants, green algae, fungi such as *Pythium* sp., *Botrytis* sp., *Fusarium* sp. and some species of cyanobacteria (Conrath *et al.*, 2004). In all these assays, oxygen concentration was maintained to <1%. However, this technique did not detect NO release when plant or its leaves are sprayed with equimolar concentrations of phosphate or even water. The technique can be extended to detect S-nitrosothiols coupled with liquid chromatography or gas chromatography (Palmieri *et al.*, 2010).

This superficially attractive method is however not often used; most likely due to the expensive equipment required (Table1).

Quantum cascade laser

Quantum cascade laser (QCL) is one of the most advanced methods for NO detection. QCL-based detector works at ambient temperature and 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 QCL lasers have recently been reviewed by us (Montilla-Bascon *et al.*, 2018), so is only briefly considered here.

For NO detection, a QC-laser emitting light at 5.3 µm (1850 cm^{-1}) is required. There are two different lasers currently being used i.e. AlInAs/GaInAs- and GaAs/AlGaAs-based materials. A gas line from the sample being analysed is based 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 setup is placed in a temperature controlled box.

One advantage of this QCL-based NO detector over chemiluminescence is possibility of long term measurements due to low ventilation rate over the sample (usually 1-1.5 L/h) 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 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 miniaturisation to make it more amenable to general laboratory use, but currently, interdisciplinary collaboration remains the best means to access and exploit this power approach to NO detection.

NO electrodes

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

Conclusions

In conclusion, in this review we have considered various means of NO. 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; most likely 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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Table 1: Advantage and disadvantages of NO methods

No	Method	Advantages	Disadvantages
1	DAF-FM and DAF-FM-DA DAF-2DA	a. Commercially available in market b. Highly sensitive, measures NO levels at nM concentrations c. Allows cellular localization studies	a. pH sensitive b. Requires oxygen c. DAF-2 does not directly react with NO d. The uptake of DAF-2 DA varies with tissue type and organelle e. Forms a N_2O_3 intermediate in the reaction f. Ascorbic and dehydroascorbic acid can react with DAF g. Accurate quantitative measurement is not possible
	MNIP-Cu fluorescent probes	a. Cheaper than DAF b. Reacts directly with NO	a. Precise quantitative

		c. MNIP-Cu probe can measure NO at anoxic, hypoxic and normoxic conditions	measurement is not possible
2	Griess reaction	a. Rapid and inexpensive b. Possible to measure NO in the gas phase	a. Poor sensitivity compared to other methods b. Quantification can be difficult c. Not applicable to cellular localization studies
3	EPR spin trap	a. NO can be measured down to picomolar concentrations b. Used properly, EPR spectroscopy is highly specific to NO	a. A semi-quantitative method requiring complex methodology to acquire and analyze the results b. Online measurement of NO is difficult

			c. Spin traps can be expensive d. Equipment is expensive and requires specialist expertise to be used properly e. Not applicable to cellular localization studies f. It cant measure NO emitted into gas phase
4	Chemiluminescence	a. Measures NO in gas phase b. NO measurement in the ppb range is possible c. Highly reliable d. Can be used to measure NO from *in vitro* enzymatic reactions, tissues, organelles	a. Difficult to measure oxidized forms of NO b. Equipment is expensive and requires specialist expertise to be used properly c. Not applicable to cellular localization studies
5	Oxyhemoglobin assay	a sensitive method,	a. Intracellular ROS

		measures NO levels In the range of 1.3-2.8 nM b. Specialized equipment is not required	can interfere with the formation of methemoglobin complex leading to false-positive results. b. Rapid pH and temperature changes in the cellular environment can affect the method's sensitivity c. Several heme-containing proteins, fall with in range of same absorbance d. Not applicable to cellular localization studies
6	Mass spectroscopy	a. Quantification of NO can be done efficiently by using ^{15}N isotopes b. In combination with MIMS/RIMS, allows the simultaneous detection of NO, O_2 and CO_2	a. Equipment is expensive and requires specialist expertise to be used properly a. Not applicable to cellular localization studies b. Expensive and

			expertise is needed
7	Quantum cascade laser	a) A very sensitive method, measuring NO down to ppb range b) Allows on line, *in vivo* NO production to be determined	a) Equipment is expensive and requires specialist expertise to be used properly b) Requires interdisciplinary approaches to analyse the results c) Can only measure NO in the gas phase d. Localization is not possible

Table1: Advantage and disadvantages of NO methods

Figure Legends:

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 intra cellular 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 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 minutes in 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 (NO scavenger) is used along with DAF FM under same incubating conditions. **c.** MNIP-Cu upon by direct reaction with NO and gives a blue fluorescence at 420 nm.

Fig. 2: Detection of gaseous nitric oxide in plants through Griess assay a) Reaction mechanism which shows that NO is first oxidised to nitrite and reacts with sulphanilamide to give a diazonium salt. The diazonium salt then reacts with N -(1-naphthyl) ethylenediamine (NED) and forms pink color azo compound showing absorbance at max 540 nm b) Set up for measurement of NO using the Griess Reagent.

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

Fig. 4: **Chemiluminescence detection of nitric oxide from plant samples**

a. Direct detection of chemiluminescence by passing released NO from plant material to reaction chamber containing ozone. NO reacts with ozone leading to formation of NO_2* which then jumps to ground state emitting light which is proportional to amount of NO produced.

b. Indirect detection of chemiluminescence by injecting plant extract into hot acidic VCl_3 where released NO passes into reaction chamber containing ozone and then signals are detected.

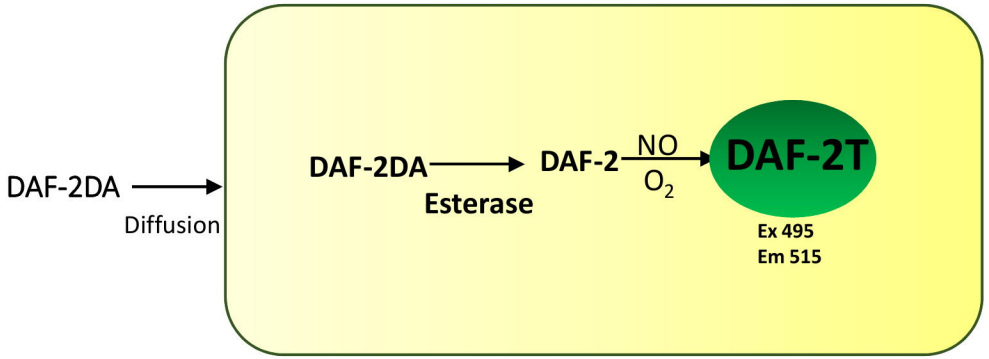
Fig. 5: A simple diagram shows the absorbance peak of methemoglobin (met-Hb) and oxyhemoglobin (Hb-O_2). The reaction of NO with met-Hb causes shift in the peak from 401 to 411 nm which indicates the oxidation of met-Hb into Hb-O_2.

Fig. 6: Schematic view of experimental setup of MIMS and RIMS based NO measurement.

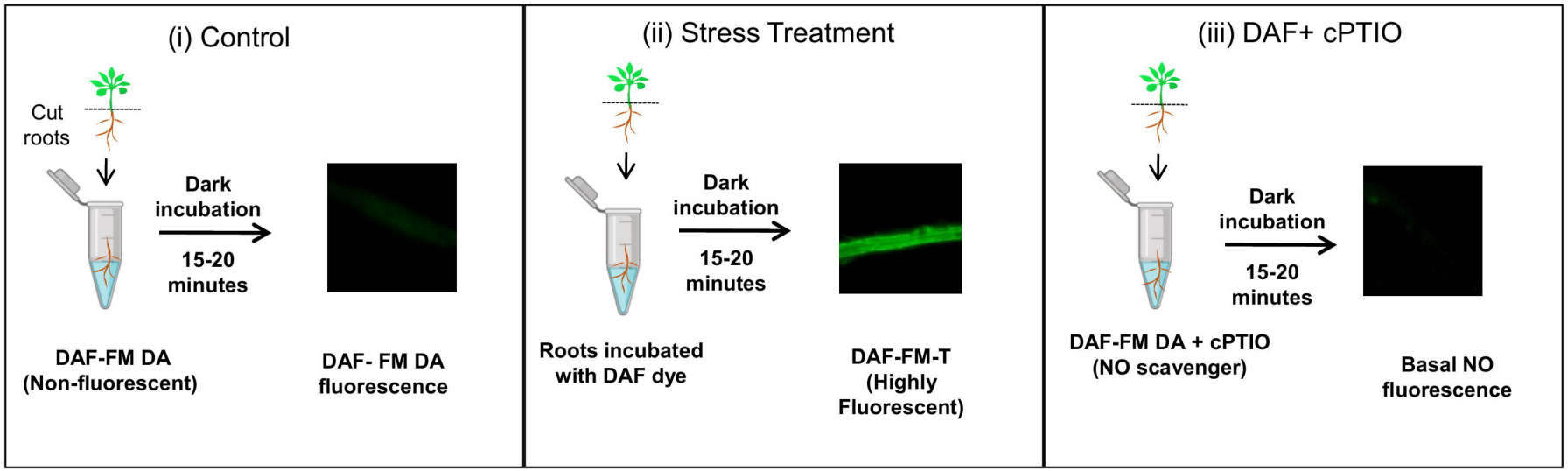
a. In MIMS, a cell suspension in an 8-10 ml reaction chamber is circulated over a thin teflon membrane by a magnetic stirrer. The dissolved gases diffuse through the membrane and pass through the ionization chamber of mass spectrometer.

b. In RIMS, sample can be a leaf of small plant or root tissue cells which fits into leaf cuvette. The produced gases pass through the capillary tube before entering to ionization chamber of mass spectrometer. A throttle valve connected to capillary tube regulates the flow of gases to ionization chamber.

Fig. 1



b.



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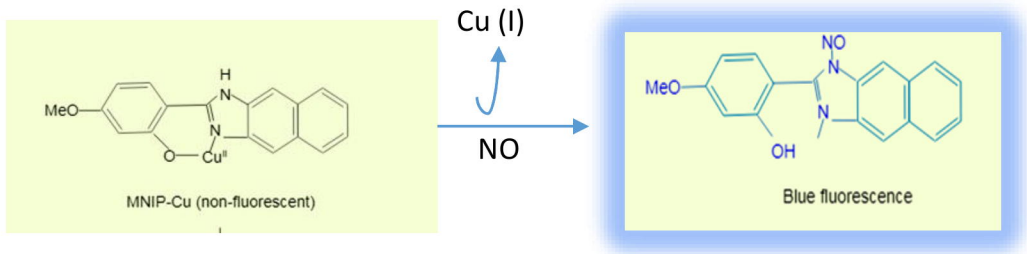

Fig 2

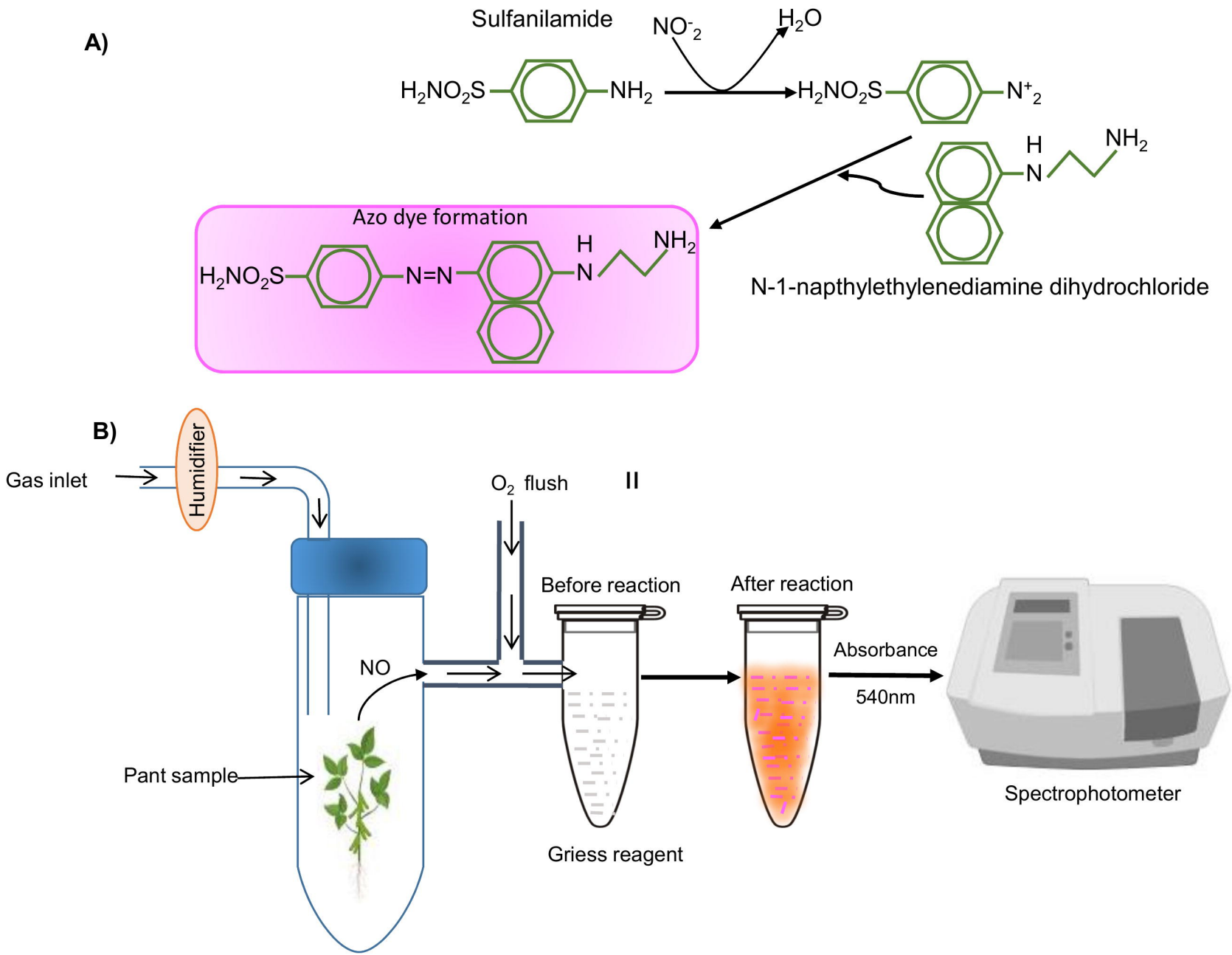

Fig 3

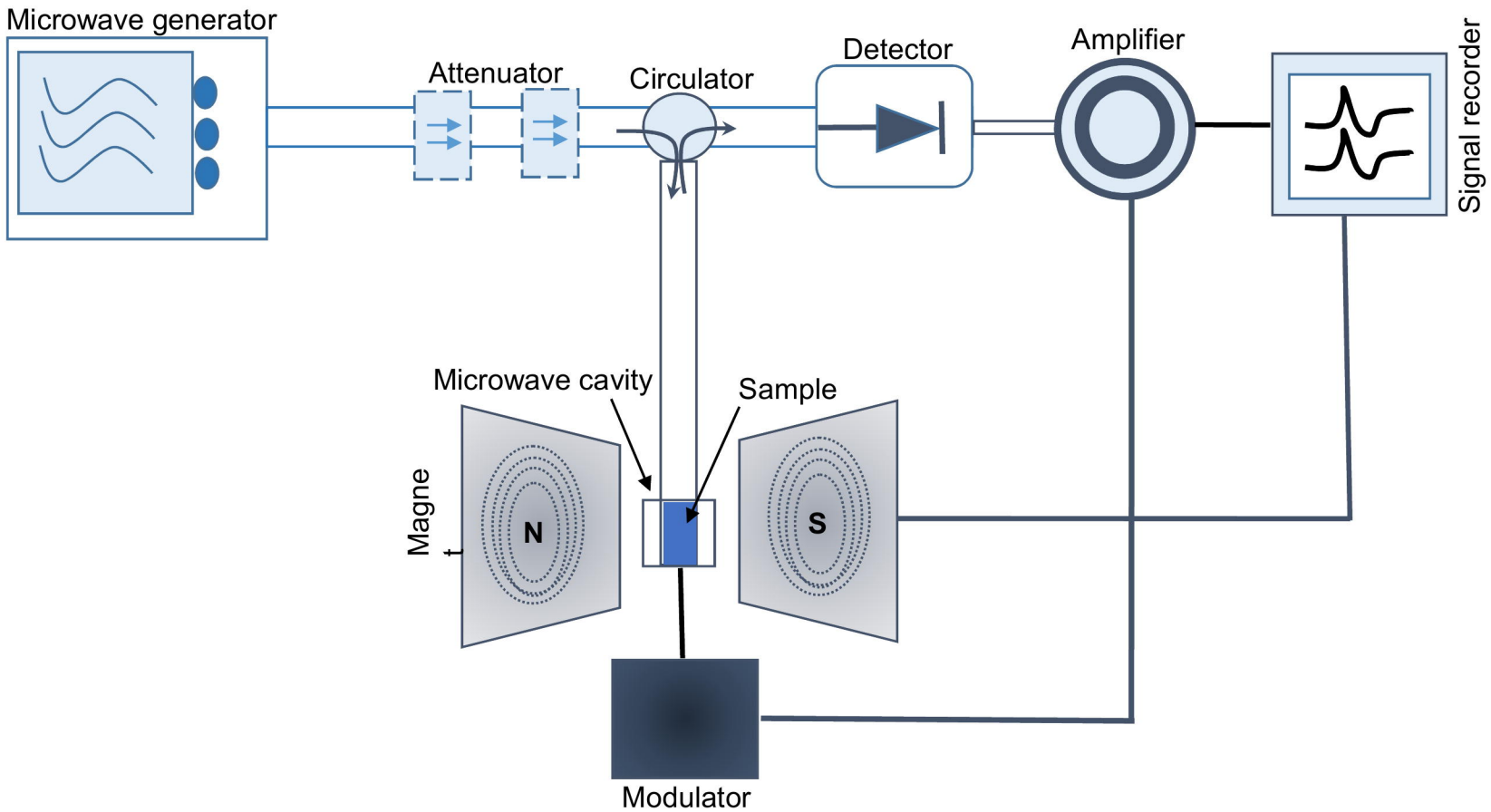

Fig 4

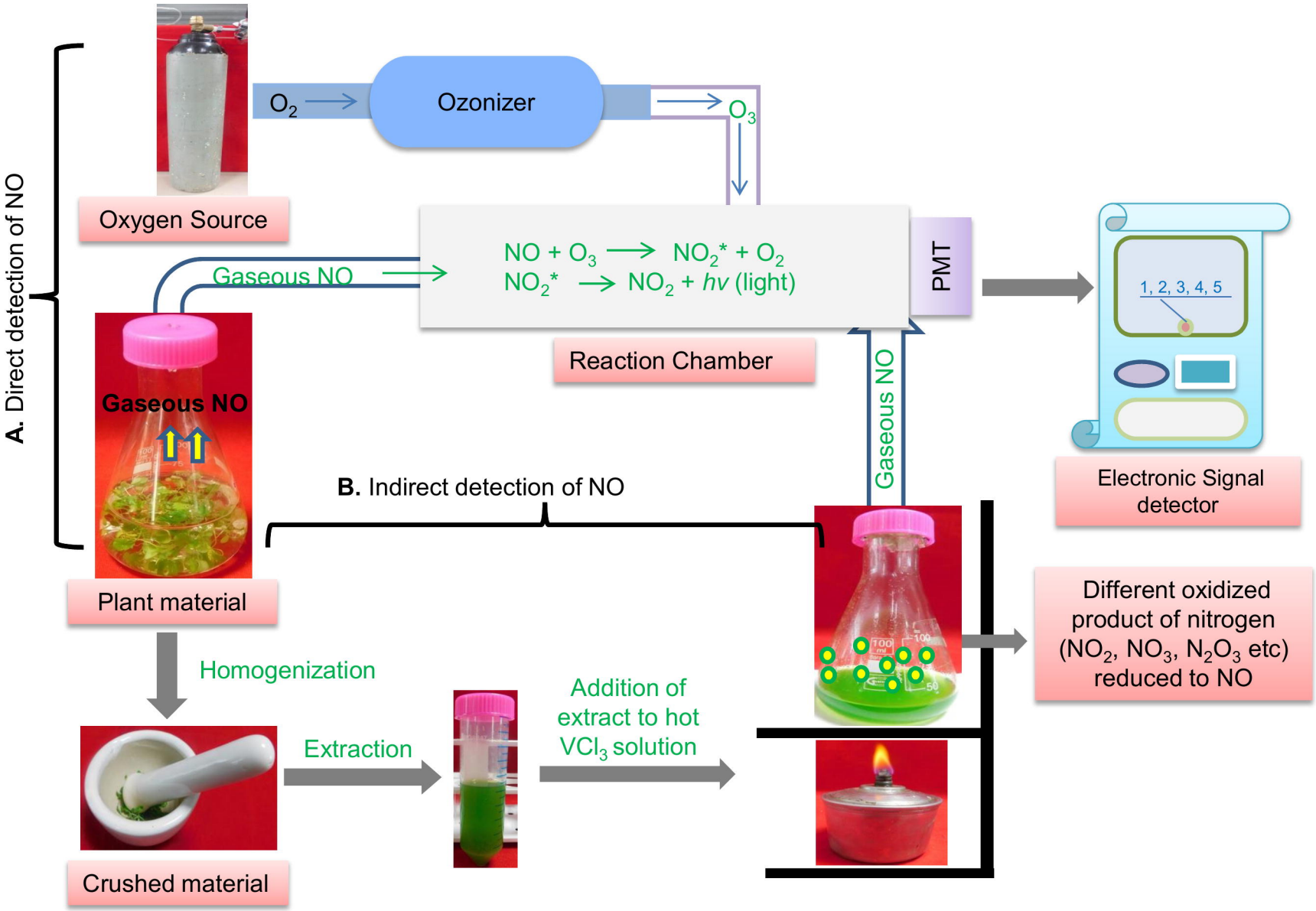

Fig 5

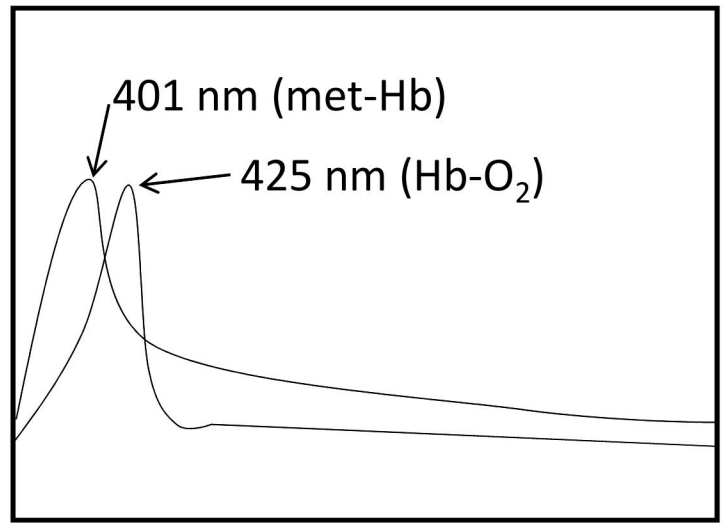

Fig 6