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TECHNICAL ADVANCE

Non-invasive online detection of nitric oxide from plants and some other organisms by mass spectrometry

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Summary

As nitric oxide (NO) is a key messenger in many organisms, reliable techniques for the detection of NO are essential. Here, it is shown that a combination of membrane inlet mass spectrometry (MIMS) and restriction capillary inlet mass spectrometry (RIMS) allows for the fast, specific, and non-invasive online detection of NO that has been emitted from tissue cultures of diverse organisms, or from whole plants. As an advantage over other NO assays, MIMS/RIMS discriminates nitrogen isotopes and simultaneously measures NO and O_2 (and other gases) from the same sample. MIMS/RIMS technology may thus help to identify the source of gaseous NO in cells, and elucidate the relationship between primary gas metabolism and NO formation. Using RIMS, it is demonstrated that the novel fungicide F 500® triggers NO production in plants.

Keywords: nitric oxide, membrane inlet mass spectrometry, restriction capillary inlet mass spectrometry, F 500®, pyraclostrobin.

Introduction

Nitric oxide (NO) is an important signaling molecule in diverse organisms (Beligni and Lamattina, 2001; Delledonne *et al.*, 1998; Durner *et al.*, 1998; Gow and Ischiropoulos, 2001; Schmidt and Walter, 1994). Despite the progress that has been made toward understanding the transduction of the NO signal in living organisms, improved methodology for the detection of NO is required. As a matter of fact, various NO assays are already available (Titheradge, 1993), but each technology displays disadvantages. Many NO assays are invasive, indirect, or unspecific, and often yield equivocal data (Michelakis and Archer, 1993; Ohnishi, 1993; Salter and Knowles, 1993; Schmidt and Mayer, 1993). For instance, the NO-mediated conversion of oxyhemoglobin to methemoglobin is prone to interference by reactive oxygen species (Murphy and Noack, 1994), whereas chemoluminescence-based detection of NO hardly detects quantitative differences in NO levels (Michelakis and Archer, 1993). Furthermore, the popular fluorescence detection of NO based on dichlorofluorescein is unable to distinguish between reactive oxygen species, peroxynitrite, and NO (Vowells *et al.*, 1995).

Recently, a spectrofluorometric assay, based on the binding of NO to 4,5-diaminofluorescein diacetate (DAF-2 DA), was shown to be suited for the direct measurement of NO from plant cells in suspension culture (Tun *et al.*, 2001). However, when monitoring spatio-temporal aspects of NO production with DAF-2 DA and confocal laser scanning (Foissner *et al.*, 2000) or epifluorescence microscopy (Gould *et al.*, 2003; Pedroso *et al.*, 2000) in plant tissue samples, these need to be wounded during sample preparation. Therefore, they might display unmeant NO emissions (Foissner *et al.*, 2000). In addition, DAF-2 DA-based

estimations of NO are often affected by differences in dye loading between different tissue types or specific organelles (Foissner *et al.*, 2000).

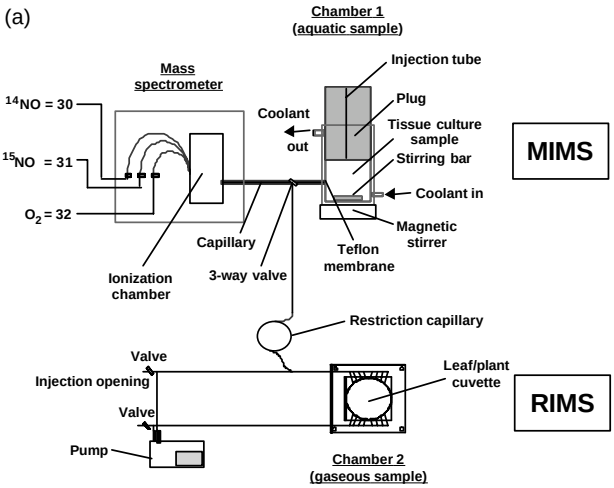
Greater accuracy in the measurement of NO must be based on direct assays, such as spin trapping electron paramagnetic resonance (Caro and Puntarulo, 1999; Huang *et al.*, 2004; Pagnussat *et al.*, 2002), photoacoustic laser spectroscopy (Leshem and Pinchasov, 2000; Mur *et al.*, 2003), or mass spectrometry (Lewis *et al.*, 1993). Eleven years ago, these authors reported the direct and simple measurement of NO emissions from mammalian cell cultures by a mass spectrometric assay referred to as membrane inlet mass spectrometry (MIMS; Lewis *et al.*, 1993). However, many biologists have glossed over this report. As a further development of the technique, we here report that a combination of MIMS and restriction capillary inlet mass spectrometry (RIMS) allows for the direct, fast, specific, and non-invasive online detection of NO from both liquid suspensions (MIMS) and the gaseous phase (RIMS).

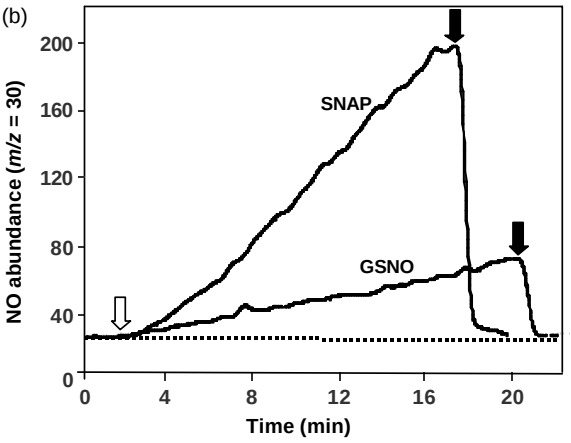
Results and discussion

MIMS/RIMS assay

In MIMS, a semipermeable membrane directly faces a cell suspension in a temperature-adjustable translucent reaction chamber (Figure 1a; Fock and Sültemeyer, 1989). Dissolved gases diffuse through the membrane into a capillary before entering the ion source of a benchtop mass spectrometer. In RIMS (Figure 1a), however, intact plant leaves or small plants are incubated in a translucent chamber that is attached to the mass spectrometer via a thin restriction capillary. In combined MIMS/RIMS assay, NO and other gases (i.e. O_2, CO_2, NO_2, etc.) from a sample pass either the membrane (MIMS) or the restriction capillary (RIMS), and then directly evaporate into the ionization chamber of a benchtop mass spectrometer, thus allowing sensitive levels of detection.

The specificity of the NO signal ($m/z = 30$) was evaluated using MIMS in three independent tests. (i) Addition of NO-releasing compounds, e.g. *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitroso-L-glutathione (GSNO), caused





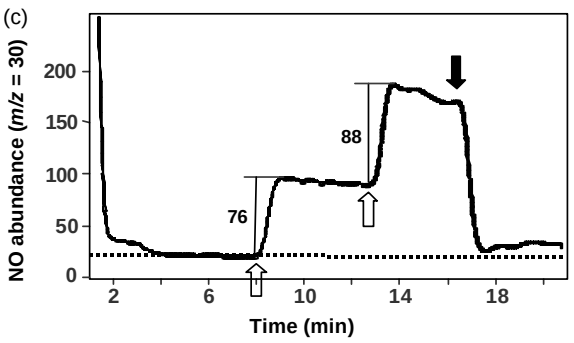


Figure 1. Schematic diagram of the experimental setup for MIMS/RIMS-based NO measurements (a), and verification of signal specificity by the addition of NO donors (b), injection of NO-saturated water (c), and scavenging of the NO signal by PTIO (b,c).

(a) In MIMS, a cell suspension in an 8–10-ml reaction chamber is circulated over a thin (50 μm) teflon membrane by a magnetic stirrer. Dissolved gases, such as NO, diffuse through the membrane and evaporate into the ionization chamber of a mass spectrometer. In RIMS, a metal bellows pump ensures rapid and efficient mixture of the 120-ml gas phase, which includes the volume of a leaf cuvette (8 cm × 8 cm × 0.4 cm). Before entering the mass spectrometer, NO and other gases pass a restriction capillary (inner diameter: 0.1 mm; length: 2 m). A three-way valve serves to switch between the two sample chambers.

(b) Changes in the abundance of mass 30 (as measured by MIMS) after addition of 180 μM of the NO-releasing compounds SNAP and GSNO into 8 ml of O_2-free buffer (HEPES/NaOH, pH 7.8). The time of injection is indicated by an open arrow.

(c) For calibration of the NO signal, the sample chamber was filled with 10 ml of water and aerated with N_2 for up to 5 min until the concentration of O_2 was nearly zero. At the times denoted by the open arrows, 5 μl of NO-saturated water (corresponding to 1.9 mM NO at 20°C) was added, resulting in a final NO concentration of 0.95 μM. Numbers give the abundance (units at $m/z = 30$) for two extremes of the received NO signal, which assigns 1 abundance unit to 10 or 13 pmol of NO.

In (b) and (c), signal specificity was further validated by the addition of the NO scavenger PTIO (150 μM) at the times indicated by the filled arrows. The dotted lines indicate zero NO.

an immediate rise in the NO signal (Figure 1b). Note that although same concentrations (180 μM) of the NO-donors were applied in Figure 1(b), the rate of NO release was about five times higher with SNAP than it was with GSNO (Figure 1b). This finding might explain the higher efficiency of SNAP to activate distinct physiological responses in various biological systems or cell types (Durner *et al.*, 1998). (ii) Injection of small aliquots of NO-saturated water into 10 ml of H_2O resulted in distinct signals of $m/z = 30$ within less than 6 sec (Figure 1c). Such a procedure can be used for simple calibration of the NO signal, which, for our system, revealed that 1 abundance at $m/z = 30$ corresponded to 10–13 pmol of NO. (iii) Addition of specific NO scavengers, such as 2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (PTIO), caused a rapid decrease in signal intensity to nearly zero NO (Figure 1b,c). Together, these data clearly show that the mass spectrometric technique allows for the specific and sensitive determination of NO, and that the system is fast enough to permit real-time measurements of changes in NO levels (Figure 1b,c).

Online detection of NO from various organisms by MIMS/RIMS assay

It has been suggested recently that various plants (Garcia-Mata and Lamattina, 2003; Rockel *et al.*, 2002; Yamasaki *et al.*, 1999), bacteria (Ji and Hollocher, 1988), and fungi (Yamasaki, 2000) can produce and then emit gaseous NO into the environment from nitrite, either via the non-enzymatic reduction of apoplastic nitrite (Bethke *et al.*, 2004), or in a side reaction catalyzed by nitrate reductase (NR; Rockel *et al.*, 2002). The NO-releasing activity of NR was facilitated at high nitrite levels and low oxygen concentrations (Rockel *et al.*, 2002). In fact, we observed considerable reduction (more than 50%) in NO yield in the presence of 21% (v/v) O_2 (data not shown). Therefore, the subsequent experiments were all performed at low oxygen concentrations (under 1%, v/v).

To validate the suitability of MIMS/RIMS to detect NO emissions from different biological sources, a variety of tissues from diverse organisms was treated with nitrite at a low oxygen level, and was assayed for the release of gaseous NO into the environment. Upon nitrate addition, extracellular NO levels continuously increased in cell cultures of mouse, higher plants, algae, and cyanobacteria, and also in suspended fungal mycelia, tobacco leaves, and whole *Arabidopsis* plants (Table 1; Figure 2).

Like most of the other techniques used to quantify NO, MIMS/RIMS allows the detection of only extracellular NO. Bearing in mind that NO is a highly active molecule and that only part of the endogenously produced NO may be released as a gas, the MIMS/RIMS technique, although being indicative, is unlikely to detect small increases in the level of intracellular NO. For this purpose, DAF-2 DA-based NO detection, combined with microscopical techniques, needs to be employed (Foissner *et al.*, 2000; Gould *et al.*, 2003; Pedroso *et al.*, 2000). This methodology, however, displays a variety of other disadvantages (see Introduction).

Table 1 Organisms for which nitrite-induced NO release has been demonstrated using adopted MIMS/RIMS assay

Organism	Assay
Mammalian cell cultures	
Mouse macrophages	MIMS
Higher plants	
Tobacco tissue culture	MIMS
Parsley tissue culture	MIMS
Soybean tissue culture	MIMS
Tobacco leaves	RIMS
Arabidopsis plants	RIMS
Green algae	
C. reinhardtii	MIMS
Fungi	
Pythium sp.	MIMS
Botrytis sp.	MIMS
Fusarium sp.	MIMS
Cyanobacteria	
Synechocystis PCC6803	MIMS
Synechococcus PCC7942	MIMS

In the MIMS assays, addition of nitrite in the absence of respective cells did not cause detectable NO production. In the RIMS experiments, spraying leaves or plants with water or equimolar concentrations of phosphate did not elicit detectable NO release. In all the experiments, concentration of O_2 was $<1\%$ (v/v).

During optimal nitrogen assimilation of plants, cytoplasmic NR catalyzes the reduction of nitrate to nitrite. The latter compound is translocated into the chloroplasts, where it is reduced to NH_4^+ by nitrite reductase. Thus, under optimal conditions, nitrite and NR do not come together in significant amounts to allow for a major production of NO by NR. However, the nitrite concentration in the cytoplasm could increase to a significant level in the absence of an electrochemical gradient across the chloroplastic envelope. This is when photosynthetic electron transport is impaired (Shingles *et al.*, 1996), for example, at night (Wildt *et al.*, 1997), or when plant cells are attacked by necrotizing pathogens (Agrios, 1997). Alternatively, at least part of the NO that is emitted from nitrite-treated plant tissues (Table 1; Figure 2) may result from the non-enzymatic reduction of nitrite in the apoplast (Bethke *et al.*, 2004).

It is remarkable that nitrite is able to induce the emission of NO also in phytopathogenic fungi (Table 1; Figure 2). Because NO can easily diffuse between cells, it is possible that in attacking fungi, plant-derived nitrite elicits the formation of NO, which might then serve to further support the plant's defense response (Delledonne *et al.*, 1998; Durner *et al.*, 1998).

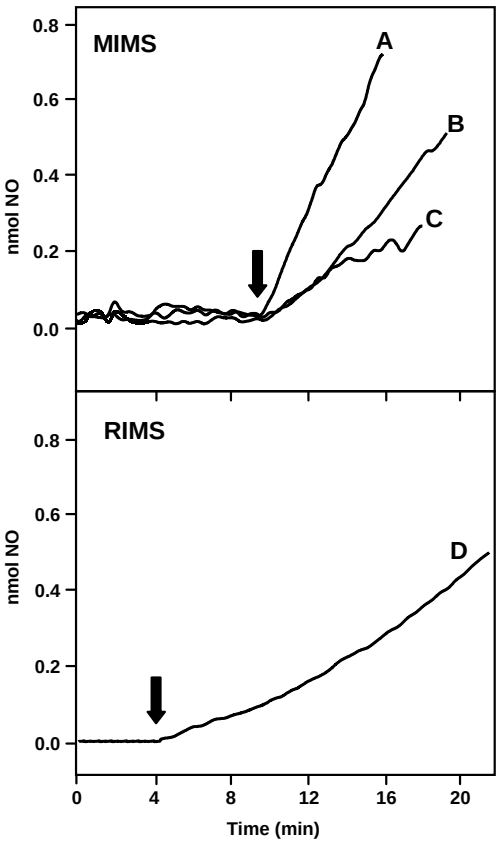


Figure 2. Validation of MIMS/RIMS-based NO assay.
A low (approximately 0.5%, v/v) oxygen level by means of nitrite (20 mM)-induces NO release in cell suspensions of *C. reinhardtii* (trace A), soybean (trace B), *Fusarium* sp. (trace C), and in a tobacco leaf (trace D). For trace D, one leaf of a tobacco plant was sprayed with 20 mM $NaNO_2$, and the subsequent NO release from the leaf was assayed using RIMS. When a sheet of paper was used instead of the tobacco leaf, emission of NO could not be detected. During the assays, temperature was kept at 20°C. Arrows denote the times of nitrite addition.

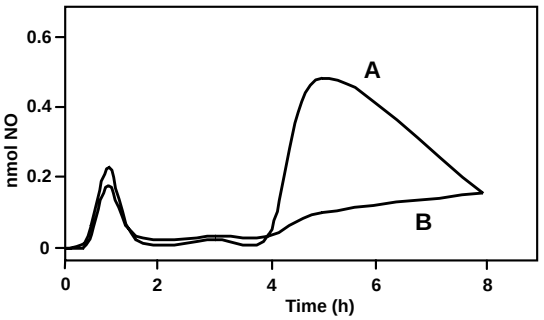


Figure 3. Nitric oxide emission in plant–pathogen interactions.
Cell suspension cultures of tobacco (trace A) and soybean (trace B) were incubated at time zero with avirulent *P. syringae* pv. *tomato* or virulent *P. syringae* pv. *glycinea*, respectively.

In addition to nitrite treatment, plant cells can also release NO when exposed to pathogens (Delledonne *et al.*, 1998; Durner *et al.*, 1998). In this case, NO likely is produced in a reaction that is catalyzed by a variant form of the P-protein of the mitochondrial glycine decarboxylase complex. Interestingly, the biochemical properties of variant P resemble those of mammalian NO synthases (Chandok *et al.*, 2003).

To investigate whether MIMS/RIMS technology is suited to also record the emission of NO in plant–pathogen interactions, suspension-cultured tobacco and soybean cells were treated with avirulent *Pseudomonas syringae* pv. *tomato* or virulent *P. syringae* pv. *glycinea*, respectively, and assayed for NO release using MIMS.

As shown in Figure 3, in the incompatible interaction between tobacco cells and *P. syringae* pv. *tomato*, there was a rapid MIMS-detectable NO burst that was maximal after approximately 1 h of treatment, and followed by a prominent second burst of NO from the 4- through 8-h time point (Figure 3, trace A). In the compatible interaction between soybean cells and *P. syringae* pv. *glycinea*, both timing and extent of the early NO burst were similar to the one detected in the incompatible tobacco–*P. syringae* pv. *tomato* interaction. The second NO burst, however, was much less pronounced (Figure 3, trace B). These results are in line with earlier findings from compatible and incompatible plant–pathogen interactions obtained using an oxyhemoglobin-/methemoglobin-based NO assay (Delledonne *et al.*, 1998). Together with these data, the results in Figure 3 support the assumption that the second NO burst is one likely key event in determining avirulence in plant–pathogen interactions.

Fungicide-induced NO release from plants and fungi

It has been speculated recently that the enhanced pathogen resistance of tobacco induced by the novel strobilurin fungicide F 500® (common name: pyraclostrobin; Herms *et al.*, 2002), as well as some aspects of the F 500®-induced 'physiological side-effect' in plants, might be because of F 500®-induced NO release (Köhle *et al.*, 2002).

MIMS analysis revealed that in tobacco cell cultures, F 500® in fact elicited a huge emission of NO after approximately 5 min (Figure 4a), which steadily increased over the 24-h detection period (Figure 4b), while respiratory O_2 uptake was dramatically inhibited (Figure 4c). In a similar manner, cultured soybean cells also exhibited F 500®-dependent NO release, which was maximal after 5 h and remained essentially unchanged over the next 19 h (Figure 4d). In intact tobacco leaves, F 500® caused an immediate emission of NO that could still be detected at the fifth day post-treatment (Figure 4e). The latter result fits with the observation that F 500® rapidly enters plant leaves where its effects last for a prolonged period (Stierl *et al.*, 2002).

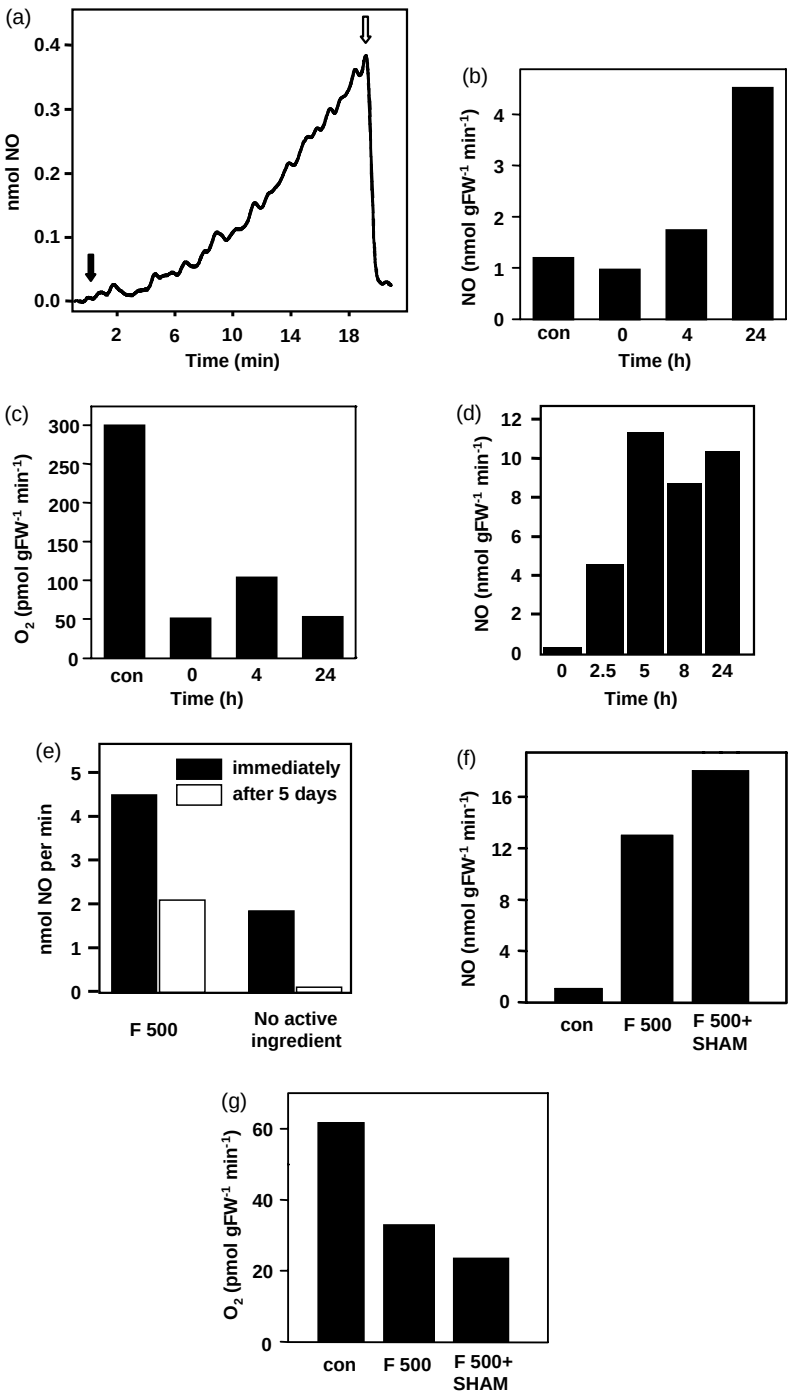


Figure 4. MIMS/RIMS assay of F 500®-induced NO release, and alterations in respiration rate of plants and fungi.
Tobacco (a–c), soybean (d), and *Botrytis* sp. (f,g) cell suspensions were treated with F 500® (5 μM, final concentration) at time zero (indicated by the filled arrow in (a)). External NO (a,b,d,f) and O_2 (c,g) were determined using MIMS. In (a), specificity of the NO signal was confirmed by addition of the NO scavenger PTIO (150 μM, final concentration) at the time denoted by the open arrow. In (b,c,f,g), con stands for control measurements with samples to which the same concentration of solvent (DMSO) was added. (e) On two tobacco plants, one leaf each was treated with an EC formulation of F 500® (0.6 mM) or with a placebo formulation void of F 500®. NO release was determined using RIMS immediately or after another 5 days. SHAM, salicylhydroxamic acid.

Interestingly, within 10 min after its application, F 500® was able to induce NO release in various fungi also (data not shown), including *Botrytis* sp. (Figure 4f). In the latter, F 500®-induced NO production was accompanied by an approximately 50% inhibition of respiration (Figure 4g). Further treatment with salicylhydroxamic acid, an artificial chemical inhibitor of alternative and F 500®-resistant respiration, led to an additional evolution of NO (Figure 4f).

Together, these MIMS/RIMS data point to NO as an important player in both F 500®-induced disease resistance and F 500®-caused 'physiological side-effect' in plants. The results obtained also indicate that fungus-generated NO could be involved in triggering plant defense responses to pathogen attack. Finally, with O_2 consumption as an example, the data show that MIMS/RIMS technology allows investigations as to the relationship between gaseous

parameters of primary metabolism and NO formation (Figure 4b,c,f,g).

$^{14}N/^{15}N$-isotope tracing

As mentioned earlier, NO in plants can be produced from nitrite either via non-enzymatic reduction in the apoplast (Bethke *et al.*, 2004), or in a side reaction catalyzed by NR (Garcia-Mata and Lamattina, 2003; Rockel *et al.*, 2002; Yamasaki *et al.*, 1999). A NR-like enzyme in the plasma membrane of root cells, which uses nitrite as a substrate (Stöhr *et al.*, 2001), a P-protein-like enzyme (Chandok *et al.*, 2003), and a recently identified plant NO synthase (Guo *et al.*, 2003) can also contribute to NO production. Furthermore, NO in plants can be produced non-enzymatically through light-mediated conversion of NO_2 by carotenoids (Millar and Day, 1997). Thus, an important issue in NO signal transduction research in plants relates to the source of NO under different physiological conditions.

With nitrite-induced NO release as the example, Figure 5 demonstrates that upon subsequent addition of ^{14}N and ^{15}N-labeled nitrite to the same tobacco cell culture, MIMS is able to distinguish between subsequently released ^{14}NO and ^{15}NO isotopomeres. Therefore, isotope tracing experiments, combined with MIMS/RIMS analysis, may be helpful in identifying the source of gaseous NO in cells. Future experiments with ^{15}N-arginine-loaded cells may provide physiological evidence for mammalian NO synthase-like enzyme activity in plants (Chandok *et al.*, 2003; Guo *et al.*, 2003).

The isotope tracing – MIMS/RIMS – combination represents a major advance in mass spectrometry-based NO detection and is an advantage over other techniques used to monitor NO production, including electron paramagnetic resonance (Caro and Puntarulo, 1999) and photoacoustic laser spectroscopy (Leshem and Pinchasov, 2000; Mur *et al.*, 2003).

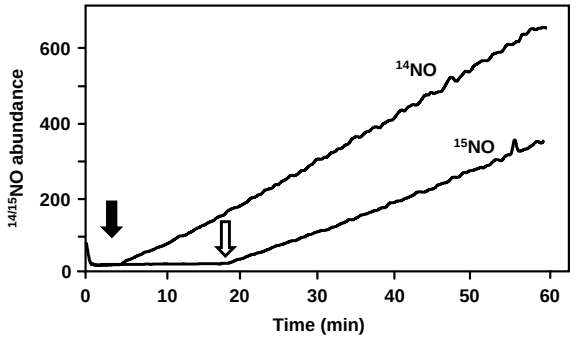


Figure 5. $^{14}N/^{15}N$-isotope tracing.
^{14}N- and ^{15}N-nitrite (each at 20 mM) were subsequently added to the same tobacco cell suspension at the times indicated by the filled and open arrows, respectively. The subsequent emission of ^{14}NO or ^{15}NO was simultaneously detected using MIMS assay. We failed to detect any NO production in the absence of cells under the conditions used.

Conclusion

Because NO is an important signal in diverse organisms, reliable techniques for its detection are required. MIMS/RIMS represents an advanced method for the non-invasive, fast, and specific detection of NO in cell cultures, tissue samples, and whole plants. The MIMS/RIMS setup is easy to handle and calibrate. Furthermore, it only requires a low-cost benchtop mass spectrometer and allows real-time measurements of extracellular NO under non-invasive conditions. Although being less sensitive than photoacustic laser spectroscopy, MIMS/RIMS technology offers a number of advantages: (i) NO measurements are possible from gaseous and liquid phases; (ii) in addition to NO, other gases (i.e. CO_2, O_2, NO_2) can be measured simultaneously, and even the analysis of more complex gases (e.g. ethylene, methyl jasmonate) is possible with the same experimental setup; (iii) isotope tracing experiments (e.g. with $^{14}N/^{15}N$) can be performed in order to elucidate the origin of extracellular NO in leaves and aquatic suspensions; and (iv) the simple handling of the MIMS/RIMS system makes it an excellent candidate for routine NO measurements, as well as for automation processes. Thus, on a long-term basis, the MIMS/RIMS technology might help to shed further light into the enigmas of the chemistry and biology of the NO key signal of life.

Experimental procedures

Materials

$^{15}NaNO_2$ was obtained from Chemotrade Inc. (Leipzig, Germany); $^{14}NaNO_2$, salicylhydroxamic acid, and PTIO were purchased from Sigma-Aldrich (Taufkirchen, Germany). F 500® was synthesized by BASF AG and provided as an EC formulation of 250 g l^{-1} (BASF Code# BAS 500 01 F). A placebo formulation void of F 500® (BASF Code# BAS 500 00 F) was used for control treatments.

Tissue cultures

Mouse macrophage cell cultures RAW264.7 (TIB-71) were purchased from the American Tissue Culture Collection, and grown at 37°C in 150-ml tissue culture flasks in HEPES (25 mM)-buffered Roswell Park Memorial Institute (RPMI) 1640 medium enriched with L-glutamine. After formation of a continuous monolayer, cells were removed from the bottom of the culture flask by gentle agitation and subjected to centrifugation (3000 ***g***, 5 min). The pellet was re-suspended in 10 ml of fresh growth medium.

Parsley, tobacco (cv. Xanthi nc), and soybean (cv. Williams 82) tissue cultures were grown as described by Kauss *et al.* (1992), Hennig *et al.* (1993), and Levine *et al.* (1994), respectively. Four to six-day-old cell cultures were washed on a funnel and suspended in fresh growth medium at a density of approximately 80 mg ml^{-1}. Fifty-milliliter aliquots of cell suspension were allowed to adapt in 250-ml Erlenmeyer flasks by shaking for at least 1 h at 120 r.p.m.

Chlamydomonas reinhardtii (strain 11–32b) was obtained from the Sammlung für Algenkulturen (Göttingen, Germany);

Synechocystis sp. PCC6803 and *Synechoccocus* sp. PCC7942 were purchased from the Pasteur Culture Collection (Paris, France). *C. reinhardtii* and the cyanobacteria were grown in high salt minimal medium (Sueoka *et al.*, 1967) and BG-11 medium (Allen, 1968), respectively. Cells were kept under continuous light at 30°C in air enriched with 5% (v/v) CO_2. Cells were centrifuged (5000 ***g***, 5 min) and concentrated in assay buffer containing 50 mM Bis–Tris-Propane/HCl (pH 8.0). Aliquots of cell suspension were adjusted to a final chlorophyll concentration of 5–10 $\mu g\ ml^{-1}$, which was determined according to Porra *et al.* (1989) before use in the experiments.

Pythium sp., *Botrytis* sp., and *Fusarium* sp. mycelia were grown in synthetic CDN (Czapek Dox Normal) medium (Cooper *et al.*, 1984) at room temperature, and were used in the assays after 7–10 days.

Plant material

Tobacco (*Nicotiana tabacum* cv. Xanthi nc) plants were grown at 22°C with 60% relative humidity in a 16-h light cycle, and used in the experiments at 6–8 weeks. *Arabidopsis* (ecotype Columbia) plants were grown for 4–6 weeks at an 8-h photoperiod and at 22°C with 60% relative humidity before use in the assays.

Bacterial pathogens

Pseudomonas syringae pv. *tomato* (strain DC3000) and *P. syringae* pv. *glycinea* (*AvrC*) were grown at 30°C in King's B medium (King *et al.*, 1954) for 1 day. After centrifugation (5000 ***g***, 5 min), bacterial cells were washed and re-suspended to approximately 35×10^6 cfu ml^{-1} in 10 mM $MgCl_2$. 1×10^7 bacteria were used for infection of tobacco or soybean cell cultures.

NO and O_2 assay

Ten-milliliter aliquots of tissue culture were transferred to the aquatic sample chamber of the mass spectrometer (model HP5970B; Figure 1). Upon respective treatments, external NO and/or O_2 was continuously monitored using MIMS.

In the experiments with intact tobacco leaves, these were inserted into the leaf/plant cuvette of the mass spectrometer (Figure 1a). The release of NO was determined using RIMS. In case of *Arabidopsis*, a whole plant was transferred to the leaf/plant cuvette of the mass spectrometer and used for RIMS analysis.

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