

Nitric oxide in plants: Investigation of synthesis
pathways and role in defense against avirulent
Pseudomonas

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*To my parents
Gowriswari and Venkata Ramana*

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Summary

During the last few years an increasing number of physiological processes in plants have been shown to be regulated by NO. NO plays important roles in growth and development, plant disease resistance, abiotic stress, and in above and underground plant organs. In recent years several enzymatic pathways and few non-enzymatic pathways were proposed for nitric oxide production in plants. The major goal of this work was to quantify NO production by plants and especially by roots, and to identify the enzymes responsible for NO production. As a major method, NO production by roots was followed through on-line measurement of NO emission into the gas phase by chemiluminescence (= direct chemiluminescence), and also by indirect chemiluminescence where trace amounts of oxidized products like NO_2^- and NO_3^- can be easily measured. Plants used were tobacco wild-type (*N. tabacum* cv *Xanthi* or cv *Gatersleben*), NR-free mutants grown on ammonium in order to prevent NR induction, plants grown on tungstate to inhibit synthesis of functional MoCo-enzymes, and a NO-overproducing nitrite reductase (NiR)-deficient transformant as well as barley, rice and pea. Induction of a hypersensitive response (HR) in tobacco leaves was achieved by using avirulent *Pseudomonas syringae* pv *phaseolicola*.

At oxygen concentrations of <1%, even completely nitrate reductase (NR)-free root tissues reduced added nitrite to NO, indicating that in roots, NR was not the only source for nitrite-dependent NO formation. By contrast, NR-free leaf slices were not able to reduce nitrite to NO. Root NO formation was blocked by inhibitors of mitochondrial electron transport (Myxothiazol and SHAM), whereas NO formation by NR containing leaf slices was insensitive to the inhibitors. Consistent with that, mitochondria purified from roots, but not those from leaves, reduced nitrite to NO at the expense of NADH. The inhibitor studies suggest

that, in root mitochondria, both terminal oxidases participate in NO formation, and they also suggest that even in NR-containing roots, a large part of the reduction of nitrite to NO was catalysed by mitochondria, and less by NR. The differential capacity of root and leaf mitochondria to reduce nitrite to NO appears to be common among higher plants, since it was observed with *Arabidopsis*, barley, pea, and tobacco. Nitrite and NADH consumption by mitochondria were also measured. Anaerobic, nitrite-dependent NO emission was exclusively associated with the membrane fraction, without participation of matrix components.

It was also examined whether root mitochondria and mitochondrial membranes produce nitric oxide (NO) exclusively by reduction of nitrite or also via a nitric oxide synthase (NOS),- and to what extent direct NO measurements could be falsified by NO oxidation. In addition to chemiluminescence, Diaminofluoresceins (DAF) were used as an NO indicators for comparison. In air, mitochondria apparently produced no nitrite-dependent NO, and no NOS activity was detected by direct or indirect chemiluminescence. In contrast, with DAF-2 and DAR-4M an L-arginine-dependent fluorescence increase took place. However, the response of this apparent NOS activity to inhibitors, substrates and cofactors was untypical when compared with commercial iNOS and is considered an artefact. With iNOS, about 2/3 of the NO were oxidized to (nitrite + nitrate). Mitochondria also appear to consume NO without increasing oxidation to (nitrite+ nitrate). We therefore assume formation of NO to a volatile intermediate (eventually N₂O₃).

It was recently shown that the hypersensitive response (HR) of tobacco triggered by the fungal elicitor cryptogein occurred independent of the presence or absence of nitrate reductase (NR). One conclusion was that NR-dependent NO formation played no role in the HR. Here we present evidence that the described scenario may be specific for

cryptogein. *Pseudomonas syringae* pv. *phaseolicola* was infiltrated into tobacco leaves from WT plant and from the NiR-deficient NO-overproducing clone 271, grown either on nitrate or ammonium. Lesion development as well as bacterial growth and sugar concentrations in leaves and in the leaf apoplast was monitored. Lesion development was positively and bacterial growth was negatively correlated with nitrate nutrition and eventually with NO formation. Bacterial growth was positively correlated with ammonium nutrition and apoplastic sugar concentrations. Total (free and conjugated) SA content were always drastically increased by bacterial infection, but there was no clear correlation with NO production. In the presence of cryptogein, *Pseudomonas* growth was drastically reduced. This shows that the assumed interdependence of bacterial growth, NO production and the HR is complex and not unifactorial.

Zusammenfassung

Die Zahl der physiologischen Prozesse in Pflanzen, die scheinbar durch NO reguliert werden, hat in den letzten Jahren stark zugenommen. NO übernimmt wichtige Rollen für die Steuerung von Wachstum und Entwicklung, für die Pathogenresistenz und bei abiotischem Stress, sowohl in unterirdischen als auch in oberirdischen Organen. In Pflanzen wurden bisher eine Reihe verschiedener enzymatischer und einige wenige nichtenzymatische Synthesewege für NO vorgeschlagen. Das Hauptziel dieser Arbeit bestand nun darin, die NO Produktion von Pflanzen und speziell von Wurzeln möglichst quantitativ zu erfassen und die beteiligten Enzyme zu identifizieren. Dieses Ziel sollte vor allem durch Chemilumineszenz-Messung von NO in der Gasphase (= direkte Chemilumineszenz) erreicht werden, aber auch durch die indirekte Chemilumineszenz, bei welcher Spuren von NO-Oxidationsprodukten wie Nitrat und Nitrit erfasst werden. Als Versuchspflanzen wurden verwendet: Wildtypen von *Nicotiana tabacum* cv. *Xanthi* oder cv. *Gatersleben*; Nitratreduktase-freie, auf Ammonium-N angezogene Mutanten, die keine Nitratreduktase (NR) induzieren; WT Pflanzen, die auf Wolframat angezogen wurden um die Synthese funktionaler MoCo-Enzyme zu unterbinden; eine NO-überproduzierende, Nitritreduktase (NiR)-freie Transformante, sowie gelegentlich Gerste, Reis und Erbsen. Eine hypersensitive Reaktion (HR) von Tabak wurde erzeugt durch Druckinfiltration von avirulenten Bakterien des Stammes *Pseudomonas syringae* pv. *phaseolicola*.

Bei Sauerstoffkonzentrationen =1% wurde exogenes Nitrit auch von völlig NR-freien Wurzeln zu NO reduziert. Folglich war NR nicht die einzige NO-Quelle von Wurzeln. Im Gegensatz dazu waren NR-freie Blattstreifen nicht in der Lage, Nitrit zu NO umzusetzen. Die NO-Bildung von Wurzeln wurde außerdem durch Hemmstoffe des mitochondrialen Elektronentransportes, Myxothiazol und Salicylhydroxamsäure

(SHAM) gehemmt, während die NO-Produktion von NR-exprimierenden Blattstreifen gegen diese Inhibitoren unempfindlich war. Damit stimmte auch überein, dass gereinigte Mitochondrien aus Wurzeln, aber nicht die aus Blättern Nitrit mit Hilfe von NADH zu NO reduzieren konnten. Die Inhibitor-Wirkung lässt darauf schließen, dass in Wurzelmitochondrien beide terminalen Oxidasen an der NO-Bildung beteiligt sind, und dass selbst in NR-haltigen Wurzeln ein großer Teil der Reduktion von Nitrit zu NO durch die Mitochondrien bewerkstelligt wird, und weniger durch NR selbst. Die unterschiedliche Fähigkeit von Blatt- und Wurzelmitochondrien zur anaeroben Nitrit:NO-Reduktion wurde nicht nur bei Tabak, sondern auch bei *Arabidopsis*, Gerste und Erbse gefunden. Sie scheint also eine generelle Eigenschaft höherer Pflanzen zu sein. Die Nitrit:NO-Reduktion wurde auch direkt als Nitrit- bzw. NADH-Verbrauch gemessen. Die Reaktion war außerdem exklusiv mit der Membranfraktion der Mitochondrien assoziiert, ohne jede Beteiligung von Matrixkomponenten.

Es wurde auch geprüft, ob Wurzelmitochondrien und- gereinigte Membranen NO ausschließlich aus Nitrit produzierten, oder eventuell auch über eine NO-Synthase (NOS). Außerdem wurde untersucht, ob und in welchem Umfang die NO-Messungen durch eine NO-Oxidation verfälscht werden konnten.

Zusätzlich zur Chemilumineszenz wurden Fluoreszenzmessungen mit Diaminofluoreszeinen (DAF) zum Vergleich herangezogen. In Luft produzierten Mitochondrien ja kein Nitrit-abhängiges NO, und eine NOS-Aktivität konnte weder durch direkte noch durch indirekte Chemilumineszenz nachgewiesen werden. Mit DAF-2 oder DAR-4M wurde jedoch eine L-Arginin-abhängige Fluoreszenzerhöhung beobachtet. Diese scheinbare NOS-Aktivität wurde mit kommerzieller iNOS verglichen und zeigte dabei sehr untypische Antworten auf NOS-Inhibitoren, Substrate und Kofaktoren. Sie wird deshalb als Artefakt beurteilt. Bei Verwendung von iNOS wurden ca. 2/3 des insgesamt produzierten NO zu (Nitrit+Nitrat) oxidiert. Mitochondrien

scheinen NO zu verbrauchen, ohne jedoch die Oxidation von NO zu (Nitrit+Nitrat) zu erhöhen. Vermutlich wird dabei ein flüchtiges Intermediat gebildet (eventuell N_2O_3).

In unserer Gruppe wurde kürzlich gezeigt, dass der pilzliche Elicitor Cryptogein eine hypersensitive Reaktion (HR) bei Tabak hervorrief, die völlig unabhängig von der Gegenwart oder Abwesenheit von NR war. Eine Schlussfolgerung daraus war, dass die NR-abhängige NO-Bildung für die HR keine Rolle spielte. Hier präsentieren wir Hinweise darauf, dass dieses Szenario Cryptogein-spezifisch sein könnte. *Pseudomonas syringae* pv *phaseolicola* wurde in Tabakblätter des Wildtyps und der NiR-defizienten, NO-überproduzierenden Mutante (clone 271) infiltriert, die entweder auf Ammonium oder auf Nitrat angezogen waren. Es wurde die Entwicklung der Läsionen, das Bakterienwachstum und die Zuckerkonzentrationen in den Blättern und im Blattapoplasten verfolgt. Die Läsionen-Entwicklung war positiv, und das Bakterienwachstum negativ korreliert mit der Nitrat-Ernährung und einer eventuellen NO-Produktion. Das Bakterienwachstum war positiv korreliert mit einer Ammonium-Ernährung und mit apoplastischen Zuckerkonzentrationen. Der Gesamtgehalt an freier + konjugierter Salicylsäure (SA) war durch bakterielle Infektion immer drastisch gesteigert, aber ohne klare Korrelation mit einer NO-Produktion. In Gegenwart von Cryptogein war das Wachstum von *Pseudomonas* fast völlig gehemmt. Diese Beobachtungen deuten darauf hin, dass die vermutete gegenseitige Abhängigkeit von Bakterienwachstum, NO-Produktion und der HR sehr komplex ist und nicht auf einfache unifaktorielle Beziehungen reduziert werden kann.

A Introduction:

It has long been known that the signal molecule acetylcholine dilates blood vessels in vertebrates. In most cases, dilation of the vessels is caused by relaxation of the muscle cells. In 1980 Furchgott discovered that an unknown substance formed in the endothelium was able to relax the smooth muscle cells and he named it as EDRF (endothelium-derived relaxing factor). Murad found that nitro-glycerine activates guanylate cyclase (GC), which produces cyclic GMP and relaxes muscle fibers. This finding then raised the question of whether nitro-glycerine was contaminated with traces of NO. Bubbling NO gas through smooth muscle cells activated GC (Arnold *et al.*, 1977) thus it was hypothesized that hormones may influence smooth muscles via NO. Few years later Ignarro showed that NO behaves exactly like EDRF (Ignarro 1989). Intensive research on NO biological functions followed worldwide. NO subsequently has been identified as a critical signal molecule in maintaining blood pressure in the cardiovascular system, stimulating host defenses in the immune system, regulating neural transmission in brain, regulating gene expression, platelet aggregation, learning memory, male sexual function, cytotoxicity and cytoprotection or development of arteriosclerosis (For review see Lamattina *et al.*, 2003).

In 1992, NO was named “Molecule of the Year” because of its wide spread biological significance and in 1998, Furchgott, Murad and Ignarro were awarded the noble prize in physiology and medicine. Twenty years back NO studies in plant systems focused on the phytotoxic properties of the oxides of nitrogen (NO_2 , N_2O_3 , NO_2^- , and NO_3^-) and their effect upon vegetation. (Rowland *et al.*, 1985). Considerable amounts of NO and N_2O are produced naturally from non polluted terrestrial ecosystems, which represent a major contribution to the natural destruction of the ozone layer (Kramlich and Linak, 1994). Ozone is produced on the surface of earth through photochemical processes

involving oxides of nitrogen, and as an air pollutant, it increases respiratory and oxidative damage in crop plants (Fehsenfeld *et al.*, 1993). In 1990 Welburn raised the question of why atmospheric oxides of nitrogen are phytotoxic and not alternative fertilizers (Wellburn, 1990). At the same time several reports showed that nitrogen gases were able to stimulate seed germination (Grubusic *et al.*, 1990, 1992)

In the last decade, the number of publications on NO in plants rose dramatically, showing that the small molecule had large effects on physiological functions ranging from stomatal closure to defense responses. Understanding how NO is produced, perceived and transduced has still is one of the major challenges in plant biology.

4. Chemistry of NO:

NO is an inorganic free radical gaseous molecule. Neutral NO has single electron in its $2p-p$ antibonding orbital and the removal of this electron forms NO^+ , while the addition of one more electron to NO forms NO^- (Stamler *et al.*, 1992). Thus the broader chemistry of NO involves a redox array of species with distinctive properties and reactivities: NO^+ (nitrosonium), NO^- (nitroxyl anion), and NO (NO radical)

i) NO^+ (*nitrosonium*). This species seems to be involved in the formation of a variety of nitroso-compounds that are generated effectively under neutral physiological conditions (Stamler *et al.*, 1992).

ii) NO^- (*nitroxyl anion*). *S*-Nitrosothiols are believed to be a minor product of the reaction of NO^- with disulfides (Stamler *et al.*, 1992).

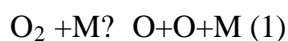
iii) NO (*NO radical*). From a biological point of view the important reactions of NO are those with O_2 and its various redox forms and with transition metal ions.

The physical half life of nitric oxide ranges from seconds to hours strongly depending on the initial concentration. The potential reactions of NO are numerous and depend on many different factors. The site and source of production, as well as concentration of

NO collectively determines whether NO elicits direct or indirect effects. To fully understand the complexity of biological effects of NO the first thing has to be considered is that plant tissues are in contact with both external (atmospheric NO and soil NO) and internally generated NO.

5. Atmospheric NO:

The atmosphere contains substantial amounts of NO. Atmospheric NO is a major green house pollutant, produced as a result of combustion of fossil fuels. The total emission rate of NO_x (NO₂, NO₃) in to the atmosphere is about 260 x10⁹ kg/year, from combustion of fossil fuels and non anthropogenic process like lightening and biological processes (Elstner and Oßwald, 1998). The mechanism for formation of NO_x from N₂ and O₂ during combustion starts by the formation of both O and N atoms at very high combustion temperatures (Manahan, 1994) or as indicated by reactions 1 and 2, where M is a highly energetic third body that imparts enough energy to the molecular N₂ and O₂ to break their chemical bonds. Once formed, O and N atoms participate in a chain reaction for the formation of NO:



Some authors observed in different ecosystems that a post-fire growth and abundance of seedlings was greater in burned areas. However, the effect of the ash generated during fires was not proven under controlled experimental conditions (Thanos and Georghiou, 1988). The smoke evolved during wildfires is the most important chemical stimulus for germination of the “fire-type” species (Brown and Van Staden, 1997). De Lange and Boucher (1990) were the first to report that plant-derived smoke stimulates seed germination but smoke-stimulated germination has been reported for many fire- and non fire-dependent species. The smoke appears to be an almost universal signal to seeds.

Interestingly, this effect could be mimicked by exogenous application of NO (Leshem, 1996; Gouvea *et al.*, 1997), and explained by the fact that NO_x species are active components of smoke (Giba *et al.*, 1999). However, atmospheric NO can affect plants; the exposure to 10 ppm of NO causes a reversible decrease in the rate of photosynthesis.

3. Biological sources of NO:

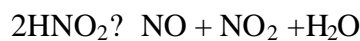
3.1 NO in soil:

Soils are an important source of NO and contribute to almost 20% of the global atmospheric NO budget (Conrad, 1995). The emission depends on soil levels of NH₄⁺ and NO₃⁻ ions (Tornton and Valente, 1996). Microbially derived N₂O and NO are products of denitrification, nitrification, and reduction of NO₃⁻ to NH₄⁺ (Colliver and Stephenson, 2000; Zumft, 1997). Prokaryotic respiratory NO₂⁻ reductases (NiRs) are able to catalyze the one-electron reduction of NO₂⁻ into NO during denitrification (Zumft, 1997). A significant increase in NO and N₂O emission from soils has been detected in soils amended with biological and inorganic fertilizers compared with non-fertilized soils (Bremner, 1997; Paul *et al.*, 1993). Soil borne NO is similar in magnitude to fossil fuel emissions of NO (Davidson and Kinglerlee, 1997). Effects of soil-borne NO on plants are largely unknown but it was proposed that NO derived from nitrogen fertilization could be responsible for the health of nitrogen-fertilized plants (Lamattina *et al.*, 2003).

3.2 Sources of NO in plants

3.2.1 Non enzymatic synthesis of NO: Non enzymatic synthesis of NO from NO₂⁻ requires low pH and could be of considerable importance since significant amounts of NO₂⁻ can be found in acidic plant tissues (Caro, 1999). The non enzymatic reduction of NO₂⁻ to NO at acidic pH values occurs in the presence of reductants such as ascorbic

acid and glutathione (Bethke *et al.*, 2004), or from protonated NO_2^- , as proposed by Yamasaki *et al.*, (1999)



It has been reported that barley aleurone layers produce NO when NO_2^- is added, probably via a non-enzymatic reduction in the acidic apoplast (Bethke *et al.*, 2004). NO_2^- entering the grain from the soil solution or released by the embryo axis, the scutellum, or the aleurone layer to the apoplast/endosperm cavity would result in an NO production that could be sensed by the other tissues (Bethke *et al.*, 2004). Soils contain NO_2^- in a concentration that can be greater than several hundred micromolar. However, values of 10–50 μM are more common in agricultural soils (Stevens *et al.*, 1998). Adequate conditions for non-enzymatic NO_2^- reduction exist in the apoplast and have been invoked to explain NO_2^- effects on germinating seeds (Giba *et al.*, 1998; Beligni and Lamattina, 2000; Bethke *et al.*, 2004).

Light-mediated conversion of nitrogen dioxide to NO can be catalysed by carotenoids although this requires an acid pH and will only occur in selected compartments in the cells (Cooney *et al.*, 1994). The formation of nitrosating agents from the reaction of carotenoids with NO_2 suggests that their ability to prevent nitrosative damage associated with NO_2 exposure in plants may be limited in the absence of light.

3.2.2 Nitrate reductase:

About 20 years ago, soybean leaves were reported to emit NO *in vivo* (Harper, 1981). Using nitrate reductase-deficient mutants of soybean, Dean & Harper (1986) found that such plants did not evolve NO, unlike wild type plants, indicating NR is a likely enzyme candidate for NO production. These workers already isolated and characterised the soybean NR activity, showing that it was NAD(P)H-dependent, had a pH optimum of 6.75, and was cyanide sensitive (Dean & Harper, 1988).

Nitrate reductase is a key enzyme of nitrate assimilation in higher plants (Pattanayak & Chatterjee, 1998; Lea, 1999), often catalysing the rate-limiting step. It uses NAD(P)H as an electron source for the conversion of nitrate to nitrite (Lea, 1999). NR also has the capacity to generate NO, an activity that has been demonstrated *in vitro* (Dean & Harper 1986) and *in vivo* (Rockel *et al.*, 2002). The enzyme generates NO from nitrite, again with NAD(P)H as an electron donor (Kaiser *et al.*, 2002). NO is probably generated using MoCo (molybdenum cofactor) as the site of catalysis, as found in another MoCo NO-producing enzyme, xanthine oxidoreductase (Harrison, 2002). However, *in vitro*, the NO generating capacity of NR could only account for a small part (< 1%) of the total NR activity extracted (Rockel *et al.*, 2002). The K_m for nitrite has been found to be approximately 100 μM , a concentration higher than the endogenous nitrite concentration estimated in illuminated spinach leaves (10 μM), and the activity was competitively inhibited by nitrate ($K_i \sim 50 \mu\text{M}$). For example, the infusion of nitrate through leaf petioles decreased NO generation. Therefore, the rate of NR-generated NO *in vivo* will be dependent on the intracellular concentrations of both these compounds, as well as on the enzymatic turnover rate of the enzyme itself (Rockel *et al.*, 2002). Intracellular nitrite has been estimated to be between 10 μM and 4.8 mM in spinach (Rockel *et al.*, 2002), while nitrate concentrations have been reported to be in the millimolar range (Miller & Smith, 1996). Using nitrite reductase (NiR) antisense tobacco, Morot-Gaudry *et al.*, (2002) showed that elevated endogenous nitrite levels caused dramatic rise in NO release, indicating a close link between nitrite and NO.

In higher plants NR is usually found as homodimer, with subunits of 100–115 kDa, depending on the species studied, although in some species it is tetrameric. The spinach NR has 926 amino acids, while others may be a little smaller, the bean NR being 881 amino acids (Hoff *et al.*, 1994). Its catalytic action requires electron transfer, a process

that involves three prosthetic groups, FAD, haem and MoCo. Kinetic analysis revealed that no single step in the electron transfer was rate-limiting (Skipper *et al.*, 2001). Interestingly, the topology of the protein reveals three structural domains, one for each prosthetic group, separated by hinge regions that are susceptible to proteolytic cleavage. Towards the N-terminus is the MoCo domain, similar to mammalian sulphite oxidase, with the haem binding region, involving histidine residues and showing similarities to cytochrome b_5 , lying in a central domain. The FAD binding site is found in the third domain, towards the C-terminal end of the polypeptide, and this domain is similar to cytochrome b_5 reductase (Campbell, 1996). Thus, like NOS, NR is composed of domains which, when cleaved from the holoenzyme have autonomous partial activity.

Expression of the NR genes is light-dependent, following a diurnal pattern, and it is nitrate-inducible (Hoff *et al.*, 1994). Light appears not only to induce the transcription of NR genes, but also to influence the protein itself, either through control of translation, or by influencing the stability of the protein once synthesised (Vincentz & Caboche, 1991).

Control of NR activity is via covalent modification, involving phosphorylation and dephosphorylation (Fig 1). NR is rapidly inactivated by phosphorylation following a light to dark transition, the site of phosphorylation being serine-543 (in spinach), an amino acid conserved in NR sequences of higher plants (Rouze & Caboche, 1992; Hoff *et al.*, 1994; Bachmann *et al.*, 1996a). Phosphorylation may be Ca^{2+} -dependent (Bachmann *et al.*, 1996a, b; Huber *et al.*, 1996). The NR phosphoprotein is recognised by a NR inhibitory protein (NIP), a member of the 14-3-3 family of controlling polypeptides (Bachmann *et al.*, 1996b; Moorhead *et al.*, 1996; Kaiser & Huber, 2001). Binding of NIP P-NR inactivates NR. The rate of NR degradation is also thought to be dependent on its phosphorylation state and association with the 14-3-3 protein (Fig 1)

Experimentally, the activity of NR has been also modulated by the addition of tungstate. Certainly, pretreatment with tungstate reduces the subsequent NR activity in cells. Tungstate serves as a molybdenum analogue, and the reduction in NR activity in plants is caused by the synthesis of an inactive tungstoprotein (Notton & Hewitt, 1971a). In fact, mRNA levels encoding NR, and levels of NR protein, are increased on tungstate treatment, although activity is diminished (Deng *et al.*, 1989). No direct inhibition of NR by tungstate has been reported. Clearly, it will be important to assess the effects of tungstate on NR activity, both *in vitro* and *in vivo*. NR can also be inhibited by cyanide (Notton & Hewitt, 1971b) or azide (Yamasaki & Sakihama, 2000), but this has limited experimental value as CN⁻ known to inhibit many other enzymes, for example cytochrome oxidases.

Undoubtedly, the identification of plants lacking NR activity, or at least with severely depleted activity, will aid greatly in the identification of the role of NR in plants – for example, the *nia1*, *nia2* *Arabidopsis* mutant, in which both NR genes are mutated (Wilkinson & Crawford, 1993).

As might be expected, NR activity in spinach leaves was reduced by the addition of phosphatase inhibitors (Rockel *et al.*, 2002). NR activity was also increased by the addition of uncouplers, while inactive NR was activated by rises in 5'AMP (Kaiser *et al.*, 1999). Along with anoxia, both 5'AMP and uncouplers led to a rise in nitrite concentration in the cells, and a rise in NO generation (Rockel *et al.*, 2002). Similar observations, showing the modulation of NR activity by 5'AMP, have also been made in cucumber (De la Haba *et al.*, 2001).

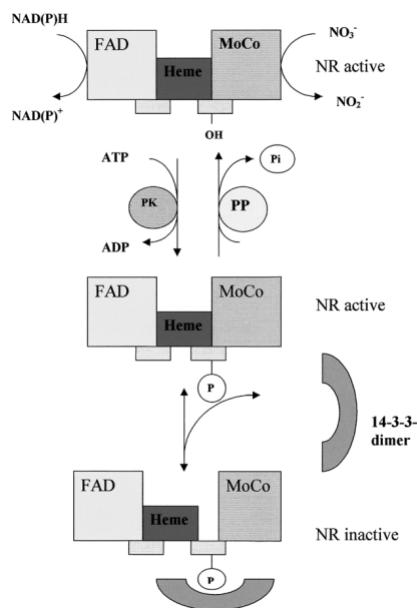


Fig 1: Model for the post-translational modulation of NR. The enzyme consists of three different functional and structural domains (labelled in different colours), which are connected by two hinge regions (in grey). The serine-543 phosphorylation site is located in hinge-1, which connects the heme and the MoCo domain. The phosphorylated serine motif is recognized by a 14-3-3 dimer which binds and, in presence of divalent cations converts NR into a completely inactive complex, which cannot transfer electrons from NAD(P)H to nitrate. This is schematically indicated by the ‘gap’ between the *heme* and the MoCo domain within the complex. Further explanations in the text (From Kaiser and Huber, 2001)

3.2.3 Nitric Oxide Synthase:

3.2.3.1 NOS in animals:

In animals, synthesis of NO is primarily accomplished by three isoforms of nitric oxide synthases (NOS) (Stuehr, 1999; Alderton *et al.*, 2001). The three isoforms of NOS are inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). The overall reaction for these enzymes is the same: NADPH dependent oxidation of L-

Arginine to N-hydroxy arginine and then to NO and citrulline. The location, regulation and of the isoforms differ however and corresponding to their respective functions.

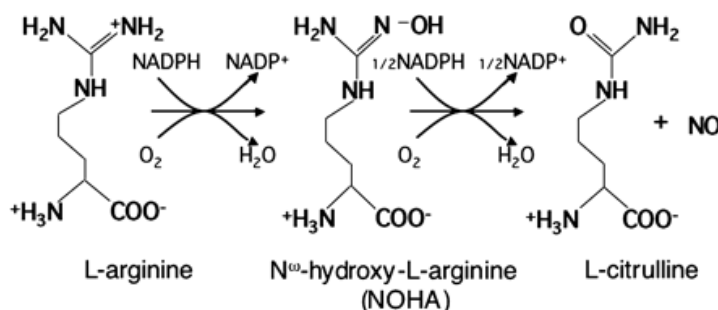


Fig 2: Reaction catalyzed by NOS. Formation of citrulline and NO from L-arginine.

The discovery of iNOS activity arose from the observation that humans and germ-free rats secrete nitrate at levels that exceed intake, and this secretion increases markedly upon infection (Green *et al.*, 1981a, b; Wagner *et al.*, 1983).

Treatment of isolated macrophages with an immunogenic elicitor (bacterial lipopolysaccharides or LPS) induced nitrate and nitrite synthesis (Stuehr and Marletta, 1985). This experiment was key as it provided a tissue culture system to study nitrate and nitrite synthesis and was used to show that L-arginine is needed by macrophages to produce inorganic nitrogen (Hibbs *et al.*, 1987; Iyengar *et al.*, 1987) and that nitrate and nitrite arise from oxidation of NO (Marletta *et al.*, 1988). These experiments established arginine as the substrate for NO synthesis.

nNOS activity was uncovered by studying the induction of cGMP synthesis in the central nervous system by the excitatory neurotransmitter glutamate (Bredt and Snyder, 1989). Glutamate functions by activating ionotropic and kainite receptors to induce a rise in intracellular Ca²⁺, which leads to the production of NO from arginine, which, in turn, activates guanylate cyclase (Ignarro, 2000).

eNOS activity was first identified by showing that vasodilator-induced production of EDRF (NO) was dependent on L-arginine but not D-arginine in perfused endothelial cells (Sakuma *et al.*, 1988). It was then shown that NO was made by extracts of endothelial cells in a Ca^{2+} -dependent manner using cGMP synthesis as an assay (Forstermann *et al.*, 1991).

The subsequent purification of NOS and, ultimately, cloning of all three isoforms provided a detailed picture of the structure and activity of these enzymes (Bredt and Snyder, 1990; Bredt *et al.*, 1991; Pollock *et al.*, 1991; Lamas *et al.*, 1992; Xie *et al.*, 1992). All three forms are bi-domain enzymes related to cytochrome P450 enzymes (Alderton *et al.*, 2001; Li and Poulos, 2005). The C-terminal oxygenase domain contains a protoporphyrin IX haem iron and tetrahydrobiopterin (H_4B) and the binding sites for arginine and oxygen. Electrons are shuttled from NADPH through the flavins to the haem and then to oxygen, which then reacts with guanidino nitrogen of arginine producing *N*-hydroxyarginine (NOHA). NOHA is oxidized further to produce NO and citrulline. Between the two domains is a site that binds calmodulin, which activates the enzyme. These enzymes vary from 130–160 kDa in size, form dimers, and are about 50–60% identical in mammals.

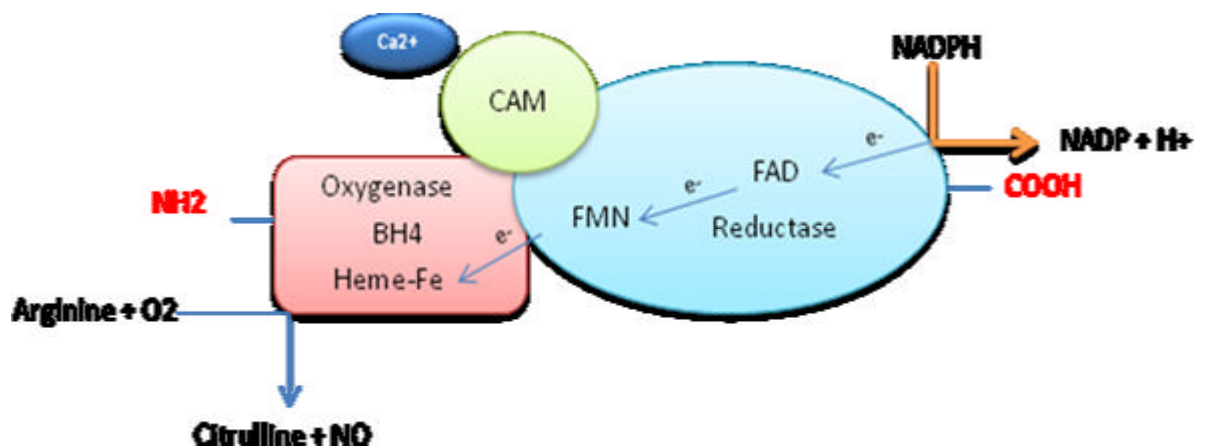


Figure 3: Schematic structure of mammalian NOS showing reductase domain and Heme-Fe domains separated by a calmodulin binding site and showing flow electrons from NADPH to Heme-Fe. (Fig modified from Crawford, 2006)

The primary differences among these enzymes are in their regulation and in their output rates of NO (Stuehr, 1999; Alderton *et al.*, 2001; Li and Poulos, 2005). iNOS binds calmodulin and needs such low levels of Ca^{2+} that it is not regulated post-translationally. Instead, its synthesis is inducible by a variety of immunological signals at the mRNA level. It produces large quantities of NO, which together with superoxide anion, serves as a cytostatic or cytotoxic agent against pathogens and tumour cells. nNOS and eNOS, however, are typically expressed constitutively and are activated by increases in Ca^{2+} levels in response to neuronal or endothelial signals. Calmodulin binds reversibly so regulation is primarily post-translational. These two enzymes are often referred to as constitutive NOS (cNOS). They produce much lower levels of NO than iNOS and are involved in signalling.

NOS enzymes are quite conserved and are found in mammals, fish, amphibians, and invertebrates (Torreilles, 2001).

No NOS gene has been found in *Saccharomyces*, but the oxygenase domain of NOS has been found in multiple species of bacteria (Adak *et al.*, 2003; Kers *et al.*, 2004), leading to the idea that eukaryotic NOS arose by fusion of a prokaryotic-like oxygenase domain to a reductase domain (Zemojtel *et al.*, 2003).

3.2.3.2 NOS in plants

In plants as well, numerous reports point on the presence of NOS-like activities (Corpas *et al.*, 2006). NOS activity was localized in peroxisomes from pea plants (Barroso *et al.*, 1999) and also in mitochondria from *Arabidopsis* (Guo and Crawford, 2005). However, the existence of NOS in *Arabidopsis*, AtNOS1, has recently been questioned (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006).

Inhibition of NO formation or of NO-dependent reactions by chemical analogs of L-arginine is usually taken as an indication that the reaction was triggered by NOS-derived NO. Immunological evidence for NOS in plants was obtained with antibodies against animal NOS (Kuo *et al.*, 1995; Sen and Chema, 1995; Barroso *et al.*, 1999; Ribiero *et al.*, 1999), but those antibodies proved to be rather unspecific (Lo *et al.*, 2000; Butt *et al.*, 2003). As no *Arabidopsis* gene or protein homolog to the large and complex animal protein has yet been found, the existence of NOS in plants is still an enigma.

More recently, a breakthrough in plant NO research was achieved by the finding of the Crawford group (Guo *et al.*, 2003; Crawford and Guo, 2005) that *Arabidopsis* contains a gene with sequence similarity to a gene from *Helix pomatia* that is implicated in NO synthesis. The gene encodes a 60 kDa protein, which, when expressed in *E. coli*, increased NO synthesis in cell extracts. When the corresponding gene (*AtNOS1*) was knocked out in *Arabidopsis*, the resulting mutant had reduced NO production in roots (measured with DAF-2DA). Contrary to animal NOS (about 140 kDa), the much smaller AtNOS1 requires no flavin or tetrahydrobiopterin, but only Ca²⁺, CaM and NADPH. AtNOS1 seems constitutively expressed. It has been suggested to be part of the signalling pathway involved in ABA-induced stomatal closure, germination, root and shoot growth, seed fertility (for review Crawford and Guo, 2005), control of flower timing (He *et al.*, 2004), senescence and protection against oxidative damage (Guo and Crawford, 2005), and seems also involved in NO production during plant–pathogen interactions, as derived from experiments with DAF-FM DA and EPR (Zeidler *et al.*, 2004; Guo and Crawford, 2005). Also, in the *atnos1* knock out mutant, induction of defence-related genes by *Pseudomonas syringae* was suppressed compared to the wild type (Zeidler *et al.*, 2004). Subsequently, two key papers expanded the role of AtNOS1 in unexpected ways. The first demonstrated that NO is a signal that regulates flower

timing by controlling the expression of flower timing genes (He *et al.*, 2004). A NO-overproducer mutant (*nox1*) was identified and shown to be an allele of *CUE*, which encodes a chloroplast phosphoenolpyruvate/phosphate translocator (Streatfield *et al.*, 1999). This mutant has higher levels of arginine and NO, and mutant flowers later than WT plants. In addition, the application of NO donors delays flowering. The *Atnos1* mutant, however, shows earlier flowering in a closed system. All these data suggest that AtNOS1, despite its different molecular properties, has functions analogous to animal NOS, but without the requirement for tetrahydrobiopterin as cofactor.

3.2.3.3 NOS in context with mitochondria:

The first report on mitochondrial localization of NOS was by Giulivi *et al.*, (1998), who detected NOS activity in purified animal mitochondria, mitochondrial homogenates, and submitochondrial particles, using EPR and oxyhemoglobin to detect NO. Indeed, NOS activity of animal mitochondria appears located in the inner mitochondrial membrane (Ghafourifar and Richter, 1997). Very recent work by Crawford's group indicates that plant AtNOS1, like the animal enzyme, is also located in the mitochondria. This view was based on the following lines of evidence:

- Computational analysis of the NOS1 protein sequence reported a high probability of being targeted to the mitochondria.
- Fluorescence from a p35S-NOS1cDNA-GFP construct strongly overlapped with MitoTracker fluorescence in mitochondria of roots and root hairs examined by confocal microscopy.
- NO production in mitochondria isolated from *Arabidopsis* WT and At- NOS1 mutant plants was detected using DAF-fluorescence (Guo and Crawford 2005) Whether AtNOS1, or (yet unknown) isoforms may be also located in other plant cell organelles, is not totally clear. Using an immunological approach, NOS-like activity in pea plants

has been reported to be localized in both peroxisomes and chloroplasts (Barroso *et al.*, 1999). The specificity of anti-NOS antibodies used for the experiments, however, has been questioned (Lo *et al.*, 2000; Butt *et al.*, 2003). Thus, at present it seems most probable that NOS-like activity in plants is exclusively located in the mitochondria. Sufficient supply of reductant in the mitochondria is assured by the citric acid cycle, and the second NOS substrate, L-arginine, may pass the mitochondrial membranes via a recently identified translocator for basic amino acids (Catoni *et al.*, 2003; Hoyos *et al.*, 2003). At this point it is also unknown whether AtNOS1 is actually exposed to the matrix side, or to the intermembrane space, as in animal mitochondria (Ghafourifar and Richter, 1997). It is also not clear whether AtNOS1 can use NADH, as well as NAD(P)H, as substrate.

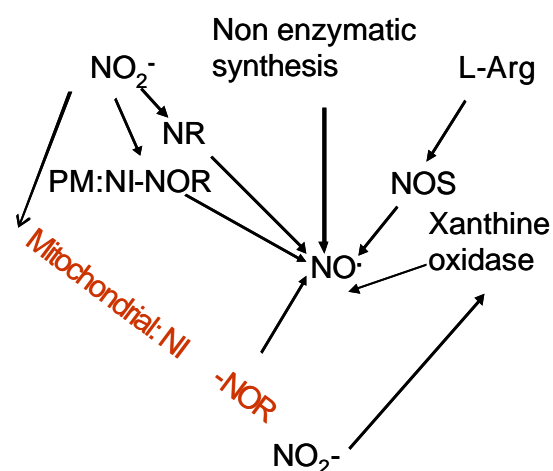


Figure 4: Enzymatic and non enzymatic pathways that contribute to NO production in plants

4 NO in plant growth and development:

Takahashi & Yamasaki, (2002) showed that NO can reversibly suppress electron transport and ATP synthesis in chloroplasts. As nitrite can be a source of NO, it was suggested that, under conditions where nitrite reduction by nitrite reductase is limited,

NR-produced NO could inhibit photosynthesis. In accordance with this suggestion, antisense-nitrite reductase (NIR) tobacco plants have been found to accumulate NO₂⁻ and have increased NO emission and exhibit reduced growth (Morot-Gaudry *et al.*, 2002).

NO is also involved in hypocotyl and internode elongation (Beligni and Lamattina, 2000). NO also increased chlorophyll content in guard cells (Leshem *et al.*, 1998). Iron mediated chlorophyll retention improved in the presence of NO (Graziano *et al.*, 2002). Several reports indicate that NO may have anti-senescence properties. Application of NO donors to pea leaves under senescence - promoting conditions decreased generation of ethylene, an endogenous driver of senescence (Leshem and Haramaty, 1996). On the other hand Magalhaes *et al.*, (2000) has shown that exposure of *Arabidopsis* plants to NO gas increased ethylene levels and that inhibition of NO synthesis did not effect ethylene accumulation. NO also increased longevity of several varieties of cut flowers (Leshem, 2001).

Fruit ripening is a senescence related process that is promoted by ethylene and can be delayed by NO. Increased ethylene production during the ripening of fruits such as banana and strawberries coincides with reduced NO emission (Leshem and Pinchasov, 2000; Leshem, 2001). NO can stimulate the seed germination in several plant species. Supply of NO donors broke dark-imposed seed dormancy in lettuce and that was reversed by application of the NO scavenger cPTIO (Beligni and Lamattina, 2000). At low pH (2.5 to 3), nitrite promoted seed germination and acidic conditions without nitrite did not (Giba *et al.*, 1998). The effects of nitrite (and thus NO) on seed germination are interesting, and suggest that the level of soil nitrite is one factor determining seed germination. NO appears also involved in root development. Auxin induced root growth and formation of lateral roots was blocked by NO scavenger cPTIO (Pagnussat, 2002).

a. Nitric Oxide and Hormones:

NO plays a central role in determining the morphology and developmental pattern of the roots. Gouvea *et al.*, (1997) provided the first evidence for participation of NO in an auxin induced process in roots. The investigators proposed that the auxin indole acetic acid (IAA) and NO might share common steps in signal transduction, because both elicit the same plant response. NO accumulation in response to auxin treatment was shown by Pagnussat *et al.*, (2002) in cucumber explants during adventitious root formation. Subsequently it was demonstrated that application of auxin to roots resulted in localized NO production and during lateral root formation, root hair formation and asymmetric NO accumulation in root tips during gravitropic response (Correa-Aragunde *et al.*, 2004; Lombardo *et al.*, 2006; Hu *et al.*, 2005). Auxin-induced adventitious, lateral and root hair formation were blocked by NO scavenger cPTIO suggesting a role of NO in these processes, moreover application of NO was able to reverse the inhibitory effects of the basipetal auxin transport inhibitor 1-naphthylphthalamic acid (NPA) (Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005). Taking together these results suggest that NO is an important molecule operating downstream of auxin through a linear signalling pathway during root growth and development. To study the contribution of NO sources in root morphological responses, pharmacological approaches using both NR and NOS inhibitors have been employed. In particular application of NR and NOS inhibitors resulted in reduced NO production and gravitropic bending. Mutants defect in NO accumulation resulted in altered NO mediated phenotypes.

An earlier report showed that activation of defense genes in tobacco by NO was also induced by cGMP (Durner *et al.*, 1998). Cyclic GMP increased in response to NO via regulation of guanylate cyclase activity (Neill *et al.*, 2003). cGMP can act via cADPR, which in turn regulates Ca^{2+} levels as was reported in various plant systems (Allen *et al.*, 1995). It was shown that the GC inhibitor inhibitor, 6-anilino-5,8-quinilinedione

(LY83583), was able to reduce AR formation in both auxin and NO-treated explants. NO can also act via a cGMP independent pathway, activating phosphatases and protein kinases including MAPKs. NO donors and recombinant NOS were shown to modulate two pathogen activated MAPKs. Auxin activates a mitogen-activated protein kinase (MAPK) signalling cascade during adventitious root formation (Pagnussat *et al.*, 2004). Thus NO regulates cyclic GMP and MAPK signalling pathways during adventitious root formation. Auxin has been reported extensively involved in regulation of cell division in plants. Auxin exerts its effect on lateral root formation by cell stimulation at the G1 to S transition (Himanen *et al.*, 2002). Auxin application induces expression of A-, B-, and D- type of cyclins as well as cyclin-dependent kinases (CDKs). Analysis of cell cycle - regulated genes showed that NO induces expression of cyclins *CYCA2;1*, *CYCD3;1* and of cyclic dependent kinase *CDKA1* during lateral root formation in tomato (Correa-Aragunde *et al.*, 2006). Interestingly Auxin - induced expression of cell cycle regulatory genes was prevented or delayed by application of the NO scavenger cPTIO, suggesting that NO is required for auxin action. In another recent report, NO was shown to stimulate the activation of cell division and embryonic cell formation in leaf protoplasts - derived cells of alfalfa (Otvos *et al.*, 2005).

Water deficit is associated with the accumulation of the plant hormone abscisic acid. ABA regulates various vital processes including seed maturation, dormancy, and vegetative growth, and induces tolerance to different stresses including drought, salinity and low temperatures (Giraudat *et al.*, 1994). Among all these processes, the control of stomatal movements of great importance for controlling water loss through transpiration stream while balancing the requirement of gas exchange for photosynthesis. Stomatal movement is effected by osmotic fluxes of water across the tonoplast and plasma membrane, such fluxes being driven by movement of K^+ and Cl^- ions through specific

channels that are activated and deactivated in response to various stimuli such as ABA (Schroeder *et al.*, 2001). Guard cell signalling is highly complex, but most signals elicit changes in cytosolic osmolarity, often in oscillating manner (Schroeder *et al.*, 2001). Calcium increases are induced by signalling molecules such as inositol trisphosphate (IP₃) and Indole hexakisphosphate (IP₆), sphingosine-1-phosphate (SIP), H₂O₂ and cyclic adenosine 5^l-diphosphoribose (cADPR) (Hetherington, 2001). Other signal transduction mechanisms in guard cells include alterations in pH, cytoskeleton arrangements, gene expression and membrane trafficking (Schroeder *et al.*, 2001; Hetherington, 2001).

ABA induces rapid NO synthesis in guard cells (and other epidermal cells) of pea (Neill *et al.*, 2002a). In pea, ABA induced NO synthesis in guard cells was required for stomatal closure, as removal of NO scavenger PTIO substantially inhibited ABA induced closure. Pharmacological evidence indicated NOS as a source of NO, as ABA-induced stomatal closure and NO synthesis were both inhibited by L-NAME (Neill *et al.*, 2002). ABA induced NO synthesis is also required for ABA- induced stomatal closure in *Arabidopsis* (Desikan *et al.*, 2002). Here, however the source of NO appears to be NR, not NOS. Tungstate strongly inhibited ABA induced NO synthesis and stomatal closure. Consistent with that, NO accumulation in guard cells and stomatal closure, both events were prevented by a NO scavenger (Desikan *et al.*, 2002). Moreover, guard cells of NO deficient *Arabidopsis nia1* and *nia2* mutant do not synthesize NO nor do they close in response to nitrite or ABA (Desikan *et al.*, 2002). It seems possible, then, that there exist species differences in NO synthesis, at least in terms of guard cell responses to ABA. It was also demonstrated that NO is an active component of H₂O₂⁻ and UV-B-induced stomatal closure (He *et al.*, 2005; Bright *et al.*, 2006). UV-B light has been reported to induce both H₂O₂ and NO in guard cells (He *et al.*, 2005).

Ethylene plays an active role in many plant responses to environmental and endogenous signals. Ethylene profoundly influences of plant growth and development, from germination and cell expansion to stress response and fruit ripening.

Evidence of the interplay between NO and ethylene in the maturation and senescence of plant tissues suggests an antagonistic effect of both gases during these stages of plant development (Leshem *et al.*, 1998) Exogenous application of NO extended the postharvest life of fresh horticultural production by inhibiting ethylene production (Leshem *et al.*, 1998). Moreover, the administration of sildenafil, an inhibitor of cGMP-degrading phosphodiesterases, results in a greater inhibition of ethylene production. This treatment prevents the wilting of flowers (Siegel-Itzcovich, 1999) indicating that cGMP could be involved in the NO-induced inhibition of ethylene production. It has been demonstrated by a non-invasive photoacoustic spectroscopic method, that endogenous NO and ethylene contents are an inversely correlated during the ripening of strawberries and avocados (Leshem, 2002).

Senescence is a process characterized by water loss and desiccation of plant tissues. As discussed above, NO can regulate stomatal closure by modulating ion channels and Ca^{2+} levels in guard cells. Therefore it would be interesting to test whether the decrease in NO levels in senescent tissue determines the loss of an important signal involved in the fine regulation of stomatal closure and a concomitant water loss that contributes to an irreversible desiccation process. More recently Durner's group has shown that S-nitrosylation inhibits one of the three methionine adenosyltransferases generating the precursor to ethylene biosynthesis (Lindermayr *et al.*, 2006)

3 NO in plant - pathogen interactions:

It has been demonstrated that NO plays an important role in plant pathogen interactions. The publication of two papers in 1998 describing the role of NO in plant defence signalling (Delledonne *et al.*, 1998; Durner *et al.*, 1998) led to a big increase in NO research. Both these papers demonstrated a key signalling role for NO during the induction of the hypersensitive response (HR). HR is a defence process activated in plants in response to pathogen attack. Associated with the HR is an oxidative burst, in which there is greatly increased ROS generation, PCD, and the activation of signalling pathways driving the expression of various defense-related genes. *Pseudomonas syringae* pv *glycinea*, induced rapid NO synthesis with kinetics similar to H₂O₂ generation (Delledonne *et al.*, 1998). Application of NO donors induced phenylalanine ammonium lyase (*PAL*) and *CHS* genes. Durner *et al.* (1998) also provided compelling evidence for the role of NO during plant defence responses. Infection of tobacco plants with HR-inducing varieties of tobacco mosaic virus (TMV) induced NOS activity that was inhibited by NOS inhibitors. NO also induced the synthesis of SA and expression of the defence-related gene *PR-1*. SA is a defence signalling molecule involved in the development of systemic acquired resistance. NO treatment of soybean cotyledons triggered the biosynthesis of phytoalexins (Modolo *et al.*, 2002). Furthermore, elicitor induced phytoalexin formation was inhibited by NOS inhibitors, implying NOS as a source of NO in this pathway. Another role of NO is in symbiosis. NO was detected in soybean nodules using EPR spectroscopy (Mathieu *et al.*, 1998). NOS immunoreactivity has been demonstrated in *Lupinus* nodules (Cueto *et al.*, 1996). However, a clear function for NO during symbiosis has not yet been established.

4 Methods to detect and measure nitric oxide:

NO is an unstable molecule that is probably produced in very low concentrations, which both make detection difficult. Significant breakthroughs in NO research have been obtained by development of new methods and technologies.

6.1 DAF

Diaminofluorescein (DAF) and its cell permeable forms (DAF-2DA, DAF-FM-DA) are most widely used fluorophores for qualitative and semi quantitative detection of NO. DAF-2 is S-nitrosated forming the highly fluorescing triazolofluorescein (DAF-2T). The diacetate has great advantage because of its efficient uptake of into living cells. DAF-2DA is readily transformed in to DAF-2 by intracellular esterase activity enabling the visualization of NO *in vivo*. DAF-2DA is a membrane permeating.

Haemogloblins or glutathione (GSH) were known to act as scavengers but low specificity for NO. The most widely used scavenger for NO is cPTIO (2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide).

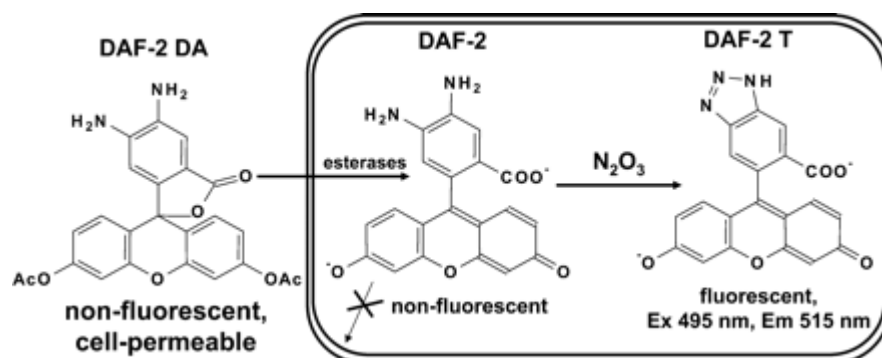


Figure 5: Fluorometric detection of NO using DAF-2DA. DAF-2DA diffuses into cells and tissue where non-specific esterases hydrolyze the diacetate residues thereby trapping DAF-2 within the intracellular space. NO-derived nitrosating agents, such as N₂O₃, nitrosate DAF-2 to yield its highly fluorescent product DAF-2T (from Tarpey *et al.*, 2004).

6.2 Electrochemical method: Commercial electrode systems are available that can be used to monitor concentrations of NO directly and continuously. They are based on the quantification of NO release in the presence of Cu^{2+} by amperometric measurements (Malinski and Taha, 1992). Example world precision instruments. Detection limit is 400 nM.

6.3 Oxyhaemoglobin assay: This method is based on oxidation of HbO_2 to methemoglobin (MetHb) by NO and the spectral change of the oxidized form (Murphy and Noack, 1994). However, as HbO_2 is also oxidized by NO_2^- , it is difficult to differentiate NO from its product of decomposition, NO_2^- . Other heme proteins such as horse radish peroxidase (HRP) have recently been employed for assay of NO. HRP is a heme protein with ferric ion; it forms a stable complex with the addition of NO that induces a spectral change from 396.5 to 420 nm. The detection limit is 10nmol L^{-1} (Kikuchi *et al.*, 1996).

6.4 Griess reagent assay : The most commonly used indirect method is the Griess reagent (Nims *et al.*, 1996), this method is based on measurement of NO_2^- , which is the stable nitrogen oxide formed following NO decomposition in aqueous solution in vitro. Analysis by the Griess reagent is based on a two step diazotization reaction and has a sensitivity limit of about 100 nmol L^{-1} .

6.5 Electron paramagnetic resonance: ESR is a direct method to detect free radicals, and some efforts have been made to detect $\text{NO}\bullet$ (Kosaka *et al.* 1992, Leshem and Harammaty, 1996). Mordvintcev *et al.* (22) employed the high affinity of $\text{NO}\bullet$ for transition metal ions to develop a method in which they used the complex of Fe^{2+} with diethyldithiocarbamate (DETC) to trap $\text{NO}\bullet$ and form a stable ternary complex $(\text{DETC})_2\text{Fe}^{2+}\text{NO}$. This ternary complex was then detected by ESR spectroscopy at 77 K. The spectral feature of the ternary complex $(\text{DETC})_2\text{Fe}^{2+}\text{NO}$ is an axial signal with an easily recognized hyperfine structure triplet at $g\bullet \hat{U} = 2.035$. However, the detection

threshold is higher and the ternary complex $(\text{DETC})_2\text{Fe}^{2+}\text{-NO}$ is not soluble in water. These drawbacks restrict this method's application in certain cases. Xu *et al.*, (2004) improved this method by extracting the ternary complex $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ from the water phase into an organic phase. By this means, the detection threshold was improved to lower than 50 nM, which was 10-fold more sensitive than the usual method. Reports on measuring $\text{NO}\cdot$ by ESR in plants are very few. Mathieu *et al.* (20) detected the spectra of leghemoglobin-NO using ESR at 77 K in soybean nodules. Modolo *et al.*, 2005 detected NO from leaf mitochondria in response to pathogen treatments.

6.6 Arginine-Citrulline assay: Some of the evidence supporting an arginine-dependent mechanism in plants comes from commercially available 'NOS assay kits' (citrulline-based assays) that measure the conversion of arginine to citrulline using ion exchange chromatography. Radiolabeled arginine is provided as a substrate, and is then separated from reaction products by cation exchange chromatography. Positively charged arginine binds the ion exchange resin but citrulline does not. The unbound (flow-through) fraction, which is generally assumed to be citrulline, is measured in a scintillation counter. Examples of this assay include the analysis of NOS activities include analysis of NOS activity in aluminium treated *Hibiscus* (Tian *et al.*, 2007), in peroxisomes of pea (Barroso *et al.*, 1999), and in elicitor treated *Hypericum* cells (Xu *et al.*, 2005). By employing the citrulline-based NOS assay, an arginine-dependent activity was discovered that was strongly stimulated by an extract of low molecular weight compounds from *Arabidopsis* leaves. Recently however, it turned out to produce argininosuccinate rather than citrulline (Tischner *et al.*, 2007). Thus the method identifies reactions that are unrelated to NOS.

6.7 Chemiluminescence

In the chemiluminescence assay NO is reacted with ozone, producing excitedstate NO₂, which upon decay to the ground state releases a photon that is detected by a photomultiplier. It is considered a useful method because of its high sensitivity and capability for real-time monitoring of NO but is limited to detection of gaseous NO (Planchet *et al.*, 2005). End-point detection of NO produced in fluids requires reduction of nitrite and nitrate to NO by vanadium (III) chloride in hydrochloric acid at 90°C. This method is ideal for monitoring NO release from plants (Rockel *et al.*, 2002). (Further details on chemiluminescence are given in methods section)

Aims of this thesis:

As pointed out above, there are several potential sources for NO in plants. Which of them really contribute, and how much it contribute in specific plant species, organs and organelles in specific situations is yet unclear. Thus it was a major task of this thesis to quantify NO production in specific tissues or organelles, and to identify contributing reactions. For that purpose, two methods were selected a) gas phase chemiluminescence and b) DAF-fluorescence

A large part of the experiments was carried out with roots from various species, and with mitochondria isolated from roots and, occasionally, from leaves. In context with the role of NO in plant defense against pathogens, tobacco (various mutants) and avirulent strain of *Pseudomonas syringae* (*pv phaseolicaola*) was used.

B Results

Chapter 1:

1.1 NO emission from roots

To measure NO emission from roots, 1g of (FW) of young roots were collected from 4 individual plants, blotted on filter paper and cut in to small segments (5mm). Segments were placed in 10ml of HEPES (pH 7.6) in a small petri dish mounted in a head space cuvette flushed with air or nitrogen (to impose anoxia).

Root segments of WT tobacco (grown hydroponically on nitrate as N-source) usually released very little or no NO in air (Figure 6A). After switching to nitrogen, however, the NO concentration in the gas stream increased, reaching a steady state after approximately 30 min, where the rate of the anoxic NO release was about $9 \text{ nmol g}^{-1} \text{ FW h}^{-1}$ (Figure 6A). The mean NR activity in corresponding root extracts was about $1 \text{ } \mu\text{mole g}^{-1} \text{ FW h}^{-1}$ (not shown). Thus, NR could produce nitrite at about a 100 fold higher rate than required for the measured NO release. Nitrate reductase (NR) –free root segments (either from a *nia 30* double mutant (Fig 6B) or from WT plants grown on ammonia as N source plus tungstate (Fig 6B), showed practically no NO emission neither in air nor in anoxia, which was originally interpreted to indicate that NR was the only NO source. But importantly, when supplied with nitrite, NO emission in anoxia was restored even in NR-free tissues (Fig 6B), consistent with our previous observations with NR-free mutants of the unicellular green alga *Chlorella sorokiniana* (Tischner *et al.*, 2004). It should be noted, that NO emission from intact root systems attached to the plant occasionally was also measured, in a specific cuvette humidified with water-saturated air. The kinetics and rates of NO emission from such intact root systems were

very similar to those obtained with root segments (not shown). However, as intact root systems were much more difficult to handle than suspensions of root segments, all experiments shown here were carried out with root segments, as in Fig 6.

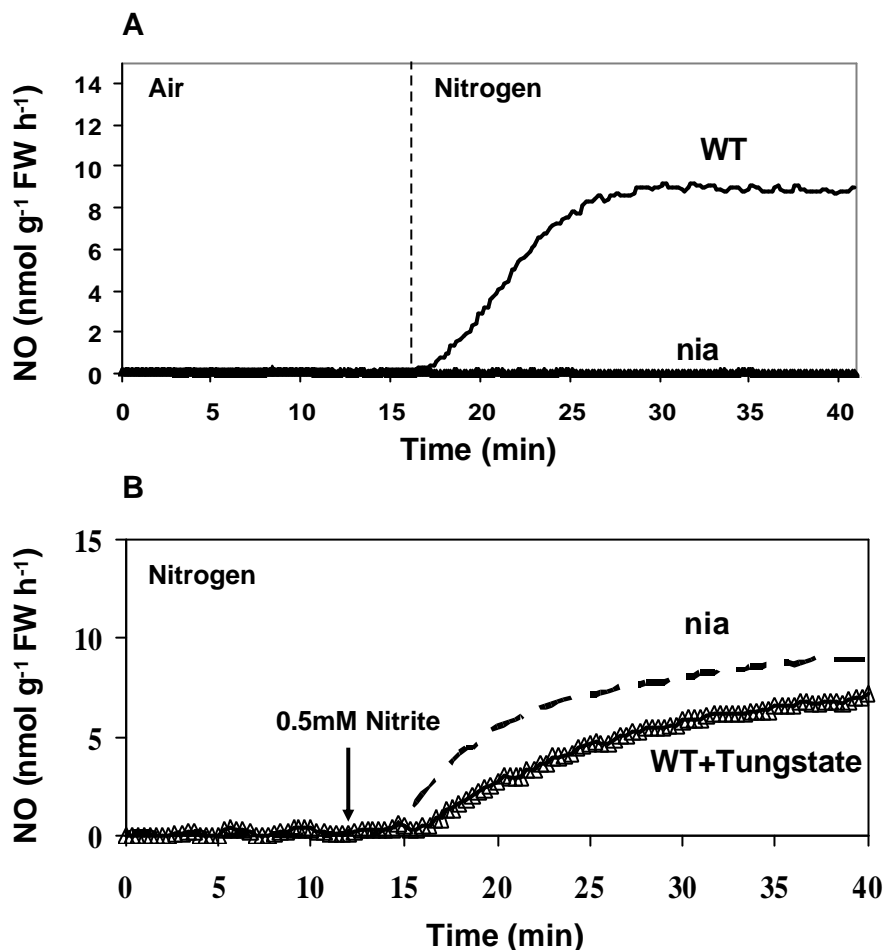


Figure 6 (A) NO emission from root segments of WT and of the NR-free *nia* 30 double mutant (both supplied with nitrite). In (B), NO emission is shown from root segments of either WT grown on ammonium plus tungstate, or from *nia* 30, after addition of nitrite. The root segments were suspended in buffer solution as described in Materials and Methods, and were flushed with air or nitrogen as indicated. The curves show representative experiments out of 3 to 8 independent experiments.

1.2 Inhibitor studies indicate possible involvement of mitochondrial electron transport:

In the experiment described in figure 6, NO emission from roots under anoxia increased and reached steady state approximately at $40 \text{ nmol g}^{-1} \text{ FW h}^{-1}$. To check involvement of mitochondria, $50 \text{ }\mu\text{M}$ Myxothiazol, a complex III inhibitor, was injected in to the solution. After Myxothiazol addition, NO emission was decreased to $10 \text{ nmoles g}^{-1} \text{ FW h}^{-1}$. To inhibit a potential alternate oxidase (AOX), 2.5 mM SHAM were added, but NO emission was not further inhibited. With 1 mM KCN, NO emission was completely inhibited. Myxothiazol and SHAM together should actually inhibit mitochondrial electron transport completely. Incomplete inhibition may therefore indicate that in WT roots NR participated in NO production from nitrite, and KCN also inhibits NR.

In order to further check the response of mitochondrial electron transport in the reduction of nitrite to NO, we used again the NR-defective double mutant of tobacco (*nia 30*) which was cultivated mostly on ammonium (hydroponics). Three days before the experiments plants were transferred in to nutrient solution containing nitrate. The mutant plants were completely devoid of NRA and contained no nitrite.

NO emission from (*nia 30*) root segments was measured as with WT. NO emission was approximately $40 \text{ nmol g}^{-1} \text{ FW h}^{-1}$ under nitrogen. After reaching steady state, $50 \text{ }\mu\text{M}$ Myxothiazol inhibited the NO emission by 75% and the AOX inhibitor SHAM (2.5 mM) caused complete inhibition as should be expected with mitochondria being the only NO source.

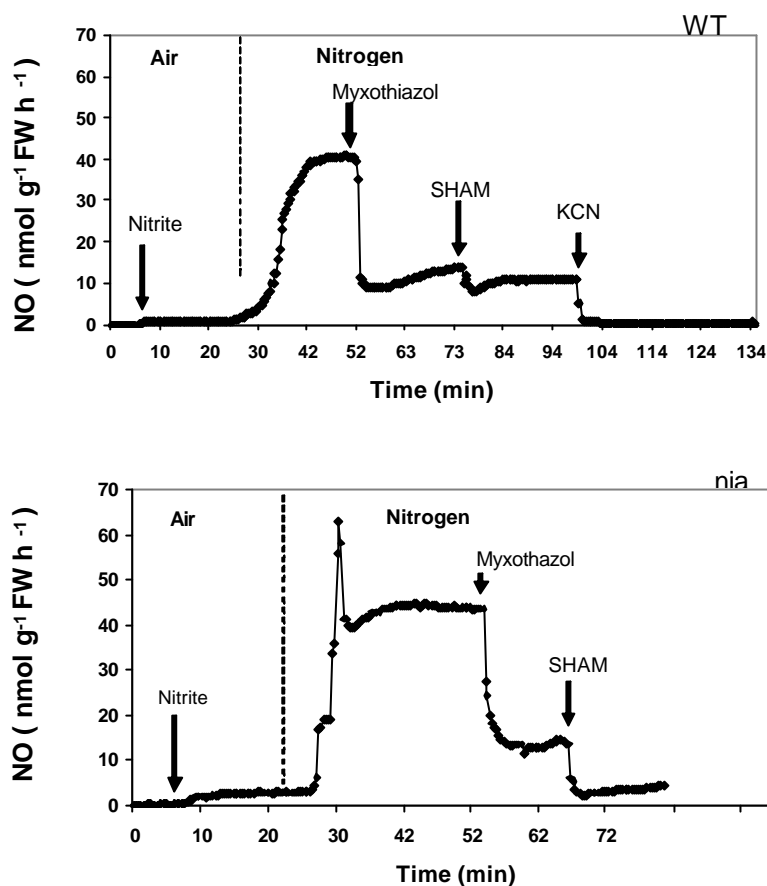


Figure 7: NO emission from tobacco root segments (a) WT or (b) *nia* double mutant lacking nitrate reductase. 1g of roots placed in 10 mL of HEPES pH 7.6. NO emission measured in air or nitrogen after addition of 0.5 mM nitrite as indicated. 50 μ M Myxothiazol, 2.5 mM SHAM or 1.5 mM KCN were added to the roots as indicated. The figures are representative of 5 independent experiments.

2 NO emission by mitochondria isolated from roots

As indicated above, tobacco roots produced NO from nitrite and this NO production was blocked by mitochondrial electron transport inhibitors. In the intact roots, such inhibitors may produce non-specific effects. In order to overcome this problem, we purified mitochondria from tobacco roots.

Ten mL of a mitochondrial suspension in a petridish were mounted in a transparent cuvette on a shaker. 0.5 mM nitrite, 1 mM NADH was added to the mitochondria and NO emission was measured under a stream of air or nitrogen. In air, mitochondria did not produce NO, but under nitrogen they produced 5 to 10 nmol mg⁻¹ protein h⁻¹. After reaching steady state, NO production was blocked by adding 20 μM Myxothazol, 2.5 mM SHAM were added (Fig 9).

To check mitochondrial activity, oxygen consumption was measured with 1 mM NADH and 0.1 mM ADP. Typically oxygen consumption of isolated mitochondria was 6-7 μmol O₂ mg⁻¹ protein h⁻¹ which compares well with literature values. As mitochondria reduced nitrite to NO this may be a signal triggering metabolic adaptation to low oxygen tensions including induction of AOX. The high rates of NO production from root mitochondria suggests that mitochondria may be more important for cellular NO production and NO signalling than previously thought.

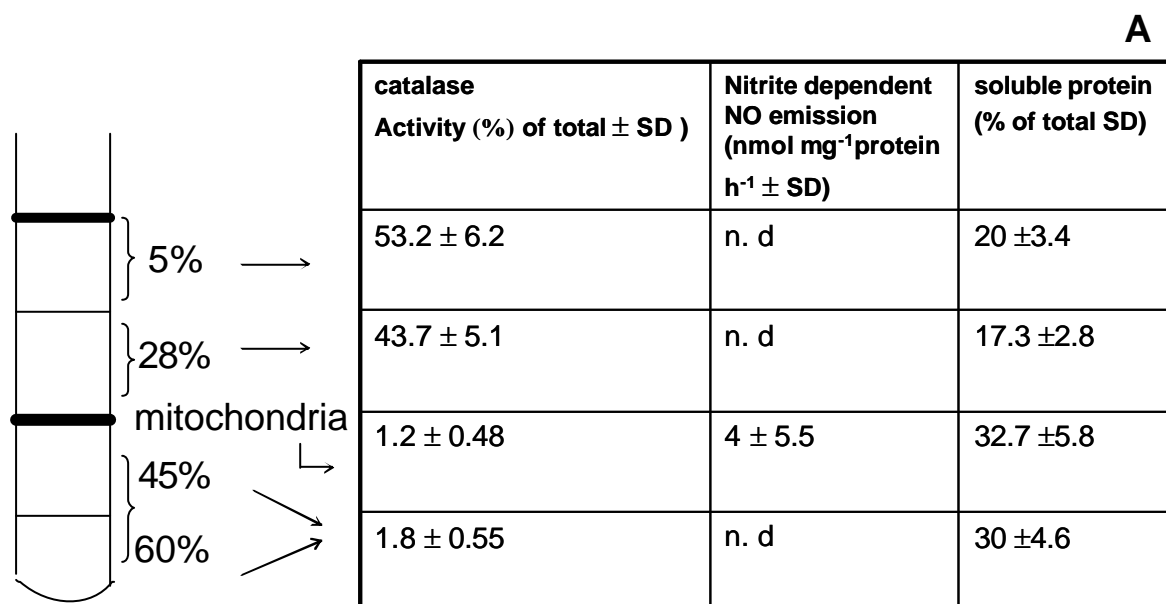


Figure 8: Characterization of Percoll fractions obtained during purification of mitochondria from tobacco roots. Catalase activity was measured a peroxisomal marker, and NO emission from nitrite plus NADH was measured as described above. It should be noted that the mitochondrial fraction was washed twice to remove Percoll, as in the

standard purification procedure, whereas all other fractions were taken directly from the gradient as indicated

Figure (8) shows the distribution of catalase activity (as peroxisomal marker enzyme) and of NO-emission of the four major fractions of the Percoll gradient used for purification of mitochondria. The mitochondrial fraction was practically free of peroxisomes, and it was the only fraction able to consume oxygen (numbers are given in the legends of various figures) and to

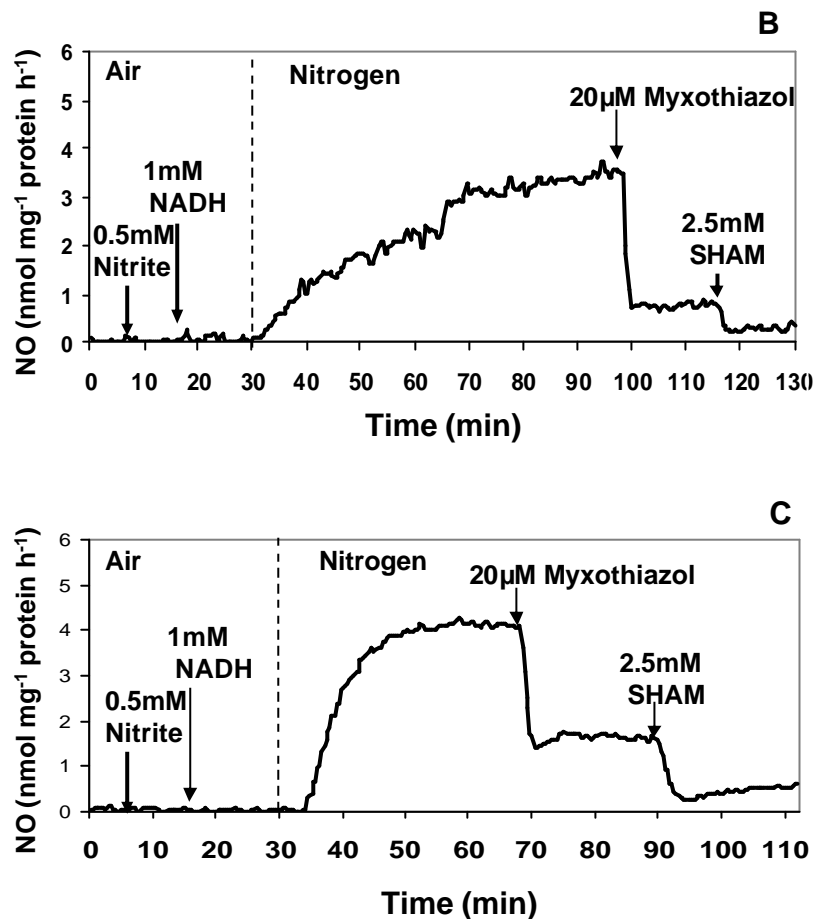


Figure 9: NO emission from tobacco root segments (b) WT or (c) *nia* double mutant lacking nitrate reductase. 1 gram of roots placed in 10 ml of HEPES pH 7.6. NO emission measured in air or nitrogen after addition of 0.5mM Nitrite as indicated. 50 µM Myxothaxol, 2.5 mM SHAM, 1.5mM KCN added to the roots as indicated. The figures are representative experiments out of 5 different experiments.

2.1 Determination of K_m for nitrite dependent NO production:

To determine K_m for NO production by mitochondria, different concentrations of nitrite (50 μM , 100 μM , 150 μM , 350 μM , 500 μM) were injected into the mitochondrial suspension in the absence of oxygen. At 500 μM , NO emission from mitochondria was close to saturation. Linear regression analysis of double reciprocal plots gave a K_m value for both mitochondria and roots of 175 μM and 210 μM , respectively.

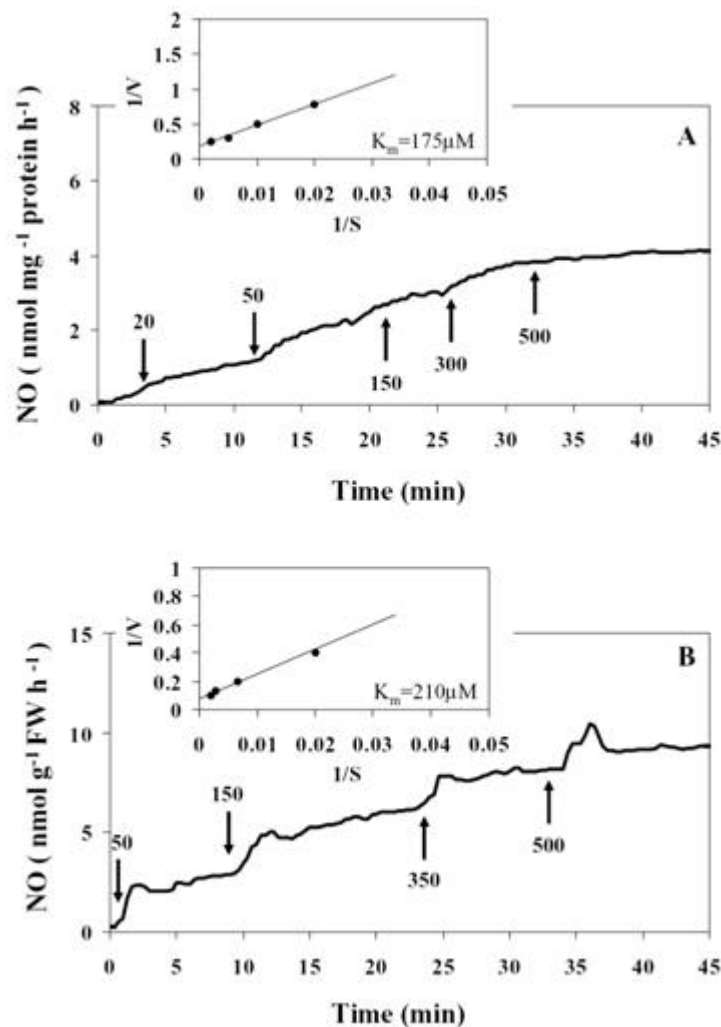


Figure 10: Nitrite affinity of anoxic NO production by isolated root mitochondria (A) or root segments (B). Nitrite (100 μL) was injected consecutively (arrows) into the

reaction mixture containing either 8 mL of a mitochondrial suspension (1.5 to 2.5 mg protein) or 1 g root segments (FW) under a stream of nitrogen. Numbers at the arrows give final (additive) concentrations of nitrite, assuming that only a small part of the preceding addition had been consumed. Oxygen uptake of mitochondria was 6 to 7 $\mu\text{mol mg}^{-1} \text{ protein h}^{-1}$. Inset gives K_m values determined by Lineweaver-Burk plots and linear regression analysis. Each NO emission curve was a representative curve out of 4 independent experiments, whereas mean values from 3 independent experiments were used to calculate the K_m .

2.2 NO emission by mitochondria isolated from tobacco suspension cells:

As already mentioned, growth of plants or cells in the complete absence of nitrate but with ammonium as N source can be used to produce plants that are completely free of NR and also of NIR. Such plants still contain other MoCo enzymes, e.g. xanthine dehydrogenase and aldehyde oxidase, which also have been suggested to produce NO from nitrite. Like NR, these enzymes are still expressed but non-functional when plants are grown on tungstate instead of molybdenum. NR free cells (ammonium cells on tungstate 100 μM) did not produce NO in air or nitrogen. However when 200 μM nitrite was added, these cells produced large amounts of NO under anoxia (see Planchet *et al.*, 2005). This anaerobic, NR - independent NO production was completely inhibited by 1mM KCN. Thus under anoxia cells were able to produce NO from nitrite independent of MoCo enzymes, confirming previous results with the *nia* double mutant.

Thus mitochondria purified from tobacco suspension cells behaved exactly like mitochondria isolated from the roots, showing nitrite to NO-reduction that was NADH dependent and sensitive to Myxothiazol and SHAM.

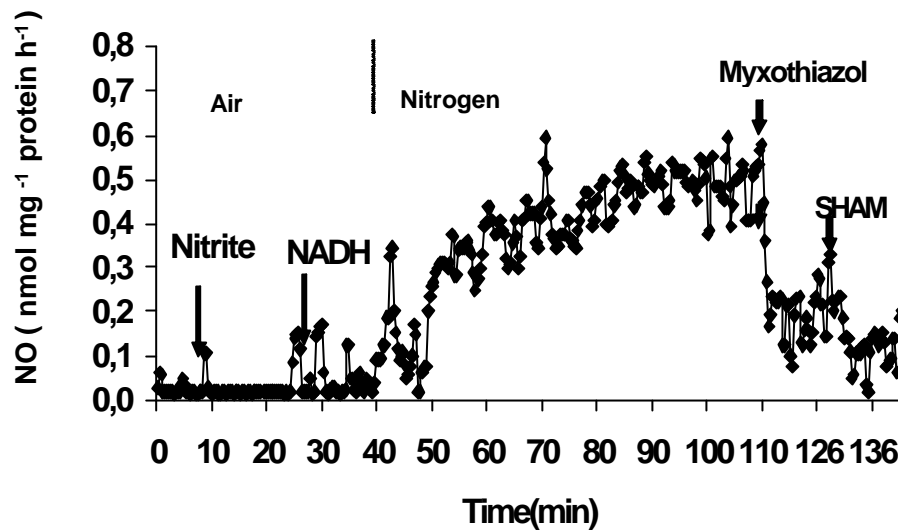


Fig 11: NO emission mitochondria (10 mL, 1.5 mg protein) of tungstate - grown cell suspensions. NO emission from 10ml mitochondria (1.5 mg protein). NO emission was measured in air or nitrogen as before. The figure is a representative example out of 6 separate experiments. Oxygen uptake of mitochondria was 4 to 5 $\mu\text{mol mg}^{-1} \text{protein h}^{-1}$.

6. NO emission from roots or mitochondria in response to different oxygen tensions:

Our previous data implied that high oxygen tensions inhibit nitrite-dependent NO emission from roots and mitochondria. In the soil environment physiological oxygen tensions for roots vary strongly coming close to anoxia under flooding conditions.

WT tobacco roots were cut in to 0.3-0.5 mm segments and 1g of roots were placed in 10 ml 20 mM HEPES pH 7.6 placed in a transparent cuvette which was under continuous stirring. Different concentrations of air mixed with nitrogen were pulled through the cuvette. With root segments of WT tobacco plants, very little NO emission was observed in 100% air. NO emission increased with decreasing concentrations of oxygen. Maximum NO emission was observed in 0% air (nitrogen).

Similarly, with purified mitochondria little NO was emission was observed in 1% oxygen then NO emission increased when oxygen concentration decreased reaching an optimum in pure nitrogen. 50% inhibition (I_{50}) of NO emission was reached at 0.05% oxygen.

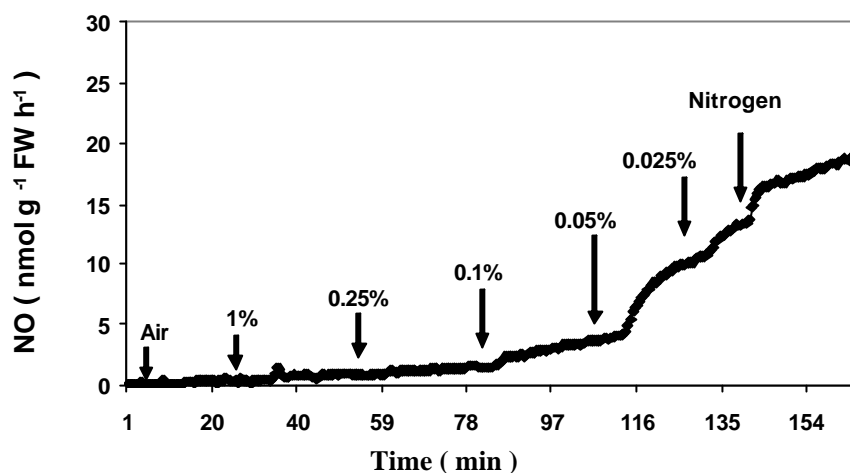


Figure 12: Effect of different concentrations of Oxygen on NO emission from WT tobacco roots. NO emission was measured from 1 g tobacco roots in 10 ml 20 mM HEPES pH 7.6, in the presence of 5 mM nitrite. Numbers indicate the percentage of oxygen present in combination with nitrogen. Total flow was 1.6 ml/min. This figure is a representative of 3 independent experiments.

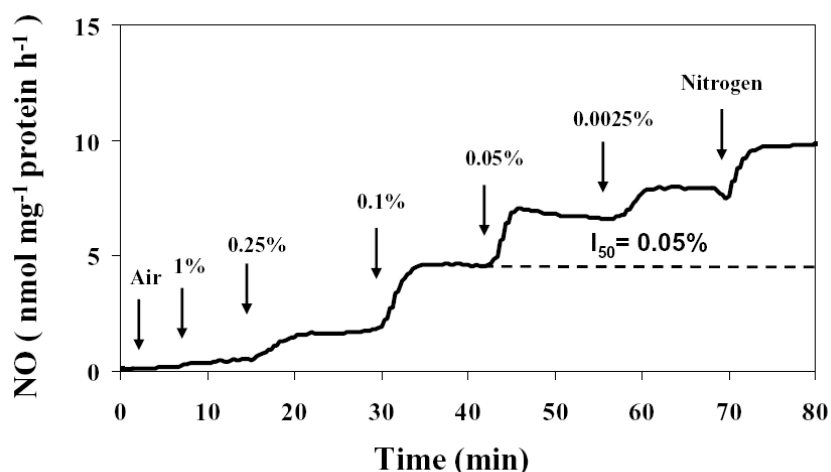


Figure 13: Oxygen response curve of NO emission from purified root mitochondria in the presence of nitrite and NADH. Reaction was started in air. Where indicated by arrows, mixtures of air and nitrogen were produced with two mass flow controllers to

give a constant total gas flow of 1.6 L min^{-1} . The resulting oxygen concentration is given as %. Nitrite was 0.5 mM , NADH was 0.2 mM . The curve is a representative experiment out of three separate experiments which produced almost identical curves.

4. NO-scavenging by mitochondria:

To check the NO scavenging capacity of mitochondria in air, a solution with a defined amount of dissolved NO (186.2 pmol) was prepared by flushing NO gas (95 ppm) into water. Aliquots (2 ml) of this NO solution were injected into vigorously stirred water. After injection, NO rapidly escaped in to the gas phase, where it was measured. At room temperature it took 20 minutes until NO emission from the solution came to an end. Integration of the emission curve revealed that recovery of injected NO was 95%. When the NO solution was injected in to 10 ml of a mitochondrial suspension only 17.5% of NO were recovered compared to water. When 2 ml of NO solution were injected into mitochondria buffer alone, 60.4% of the NO were recovered from the buffer compared with water. Thus the mitochondria buffer alone scavenged some NO.

To check the involvement of oxygen in NO scavenging, identical experiment were conducted under nitrogen (Fig 14). In pure water, 95% of added NO was recovered. However when NO was injected in to 10 ml of a mitochondrial suspension under nitrogen stream, about 27.4% were NO recovered. When NO injected in to 10 ml of mitochondria buffer, 66% of NO recovered. This indicates little oxidative component in NO scavenging.

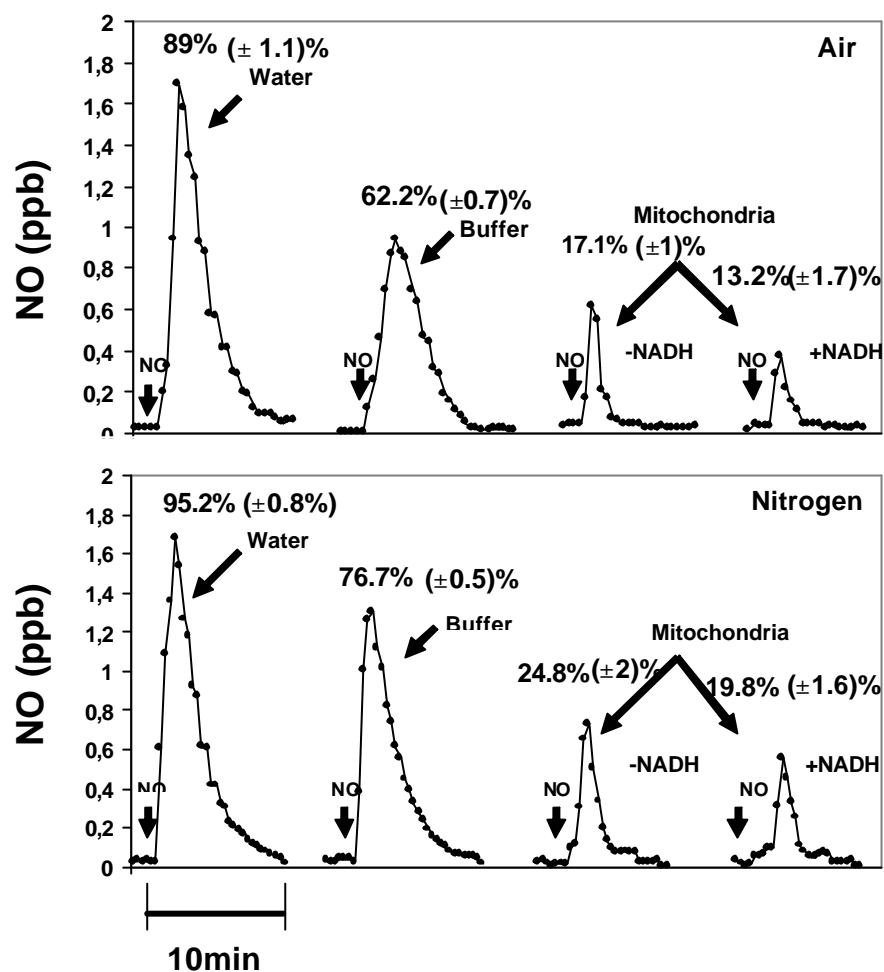


Figure 14: Effect of oxygen (a) and nitrogen (b) on the recovery of NO from water, buffer or a suspension of tobacco root mitochondria in the presence and absence of respiratory substrate (NADH). NO emission was followed after injection of 2 ml of NO solution into 10ml of water, or buffer (0.3 M Sucrose, 20 mM HEPES pH 7.6, 2 mM $MgCl_2$, 1 mM EDTA, one proteinase inhibitor tablet) or 10 ml mitochondria, in the presence or absence of 1 mM NADH. Numbers give the amount of NO recovered as percentage of theoretically added NO. 10 ml of mitochondria in the cuvette contained 1.5 to 2.5 mg protein. Oxygen consumption of the mitochondria was $6-7 \mu\text{mol O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$. Numbers (%) are means average \pm SD from 5 experiments.

To determine the effect of the mitochondria concentration on NO scavenging, NO solution was added to 10 ml solution containing 1.5 ml, 3 ml, or 5 ml of mitochondria. NO scavenging was obviously dependent on the concentration of mitochondria, increasing with the mitochondrial concentration (Fig 15).

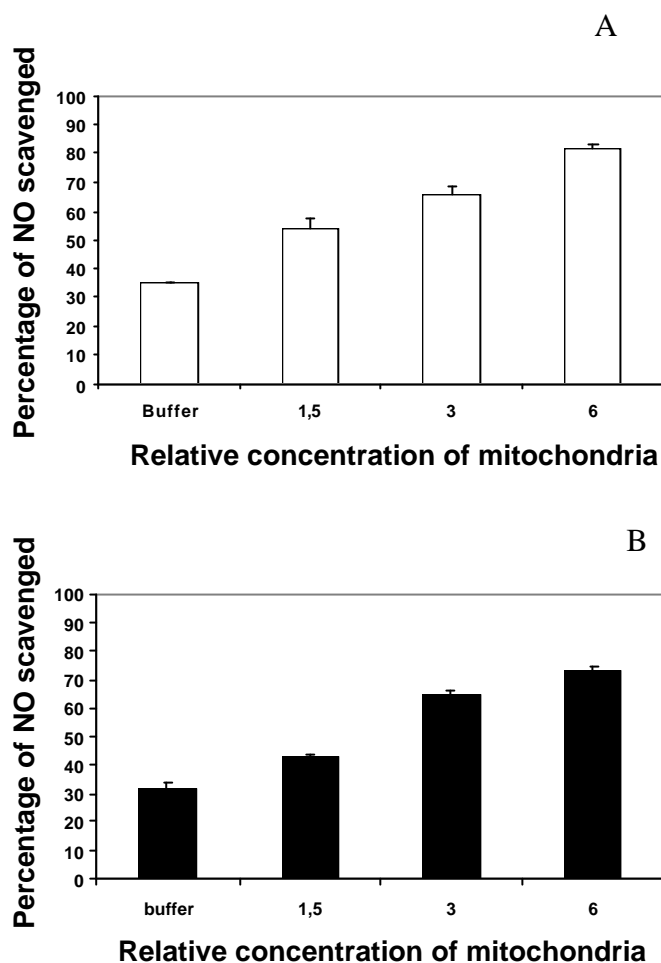


Figure 15: Dependence of NO scavenging on mitochondria concentration in (A) air or (B) nitrogen. NO emission was followed after injection of 2 ml of NO solution in to buffer (0.3 M Sucrose, 20 mM HEPES pH 7.6, 2 mM $MgCl_2$, 1 mM EDTA, and one proteinase inhibitor tablet) or different dilution of mitochondria with buffer. Numbers give amount of NO scavenged as percentage of theoretically added NO. Mean (\pm) SD of 3 replicate experiments.

In order to determine whether NO scavenging by mitochondria (in air or nitrogen) was dependent or independent on electron transport the experiment of Fig was repeated with or without added NADH (1 mM). Interestingly mitochondria with NADH performed 25% more NO scavenging (Fig 14) compared to mitochondria without NADH. This response was same in air and nitrogen. In buffer without mitochondria NO recovery was the same with and without NADH.

The reason for the higher mitochondrial NO scavenging with NADH is not known. Formation of superoxide in actively respiring mitochondria could be one reason since

NO reacts with certainly superoxide which may contribute to scavenging. However, the NADH effect was visible also in nitrogen, where superoxide should not be formed.

5. NO emission from leaf mitochondria:

Previously our group demonstrated NO production from leaves (Planchet et al., 2005). During darkness in air, detached tobacco leaves, with petioles in nitrate solution (10 mM), emitted 5 times less NO than in light. Highest NO emission was detected when leaves were kept in dark under a stream of nitrogen, which was more than 1000 times higher than in air (dark).

Under anoxia, leaf slices from nitrate-grown WT plants produced NO at similar rates (on a FW basis) as root segments. But in contrast to roots, this NO emission was insensitive to Myxothiazol (Fig 16). Leaf slices of *nia 30* did not emit NO even when fed with 0.5 mM nitrite under anoxia (Fig 16). Both findings strongly suggest that in leaf tissues, all NO is derived from NR, suggesting that, leaf mitochondria are not able to produce NO.

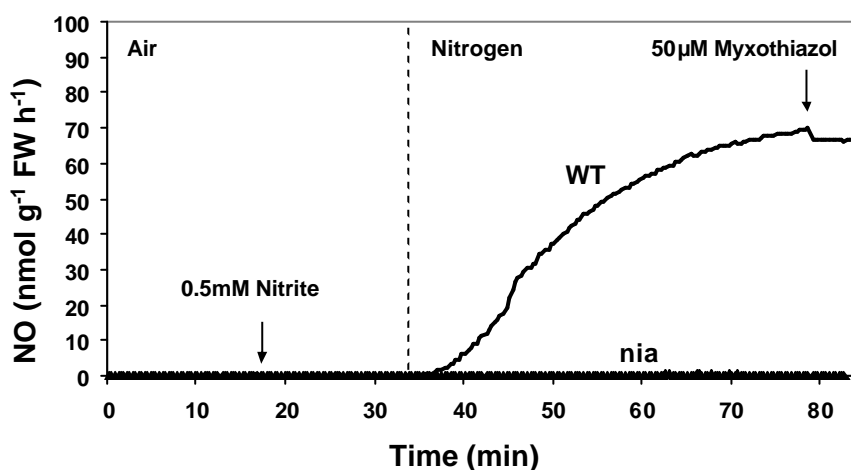


Figure 16: Comparison of NO emission from tobacco leaf slices from WT and from the NR -free *nia* leaf slices. Myxothiazol was added were indicated by an arrow. Curves are representative recordings out of at least 3 similar independent experiments.

In order to check the participation of mitochondria in NO emission by leaves, mitochondria were isolated from tobacco leaves of both, WT and of the nitrate reductase deficient double mutant *nia 30*. 10ml of almost chlorophyll free mitochondria were placed in a small petridish in a transparent cuvette placed on a magnetic stirrer. The cuvette was covered with black cloth in order to avoid any light effects. Generally leaf mitochondria produced much less NO than root mitochondria. In air, NO emission was practically absent and even in nitrogen the rates remained very low. As Myxothiazol and SHAM caused only little inhibition of NO formation, we suggest that in leaves, NR was the major source for NO, quite in contrast to the roots.

In order to check whether the above described differential function of leaf and root mitochondria was unique for tobacco, we examined nitrite-dependent NO production of other species, both with leaf and root slices as well as with mitochondria purified from leaves and roots. In Fig. 17, leaf slices from nitrate-grown barley and pea produced even higher amounts of NO under anoxia than tobacco leaf tissues. Leaf slices from ammonium-grown plants, which do not express NR in leaves, emitted almost no NO even when fed with nitrite (Fig 17) Consistent with that, mitochondria purified from young pea or barley leaves emitted only very little NO when fed with nitrite plus NADH (Fig 17 B and D).

In contrast to leaves, root segments from barley (Fig 18 A) and pea (Fig 18 C) grown on ammonium produced NO at rates comparable to those of tobacco root segments, with slightly different kinetics between barley and pea. Importantly, unlike leaf mitochondria, root mitochondria purified from barley (Fig 18 B) and pea (Fig 18 D) also emitted NO when fed with nitrite and NADH, just like root mitochondria from tobacco.

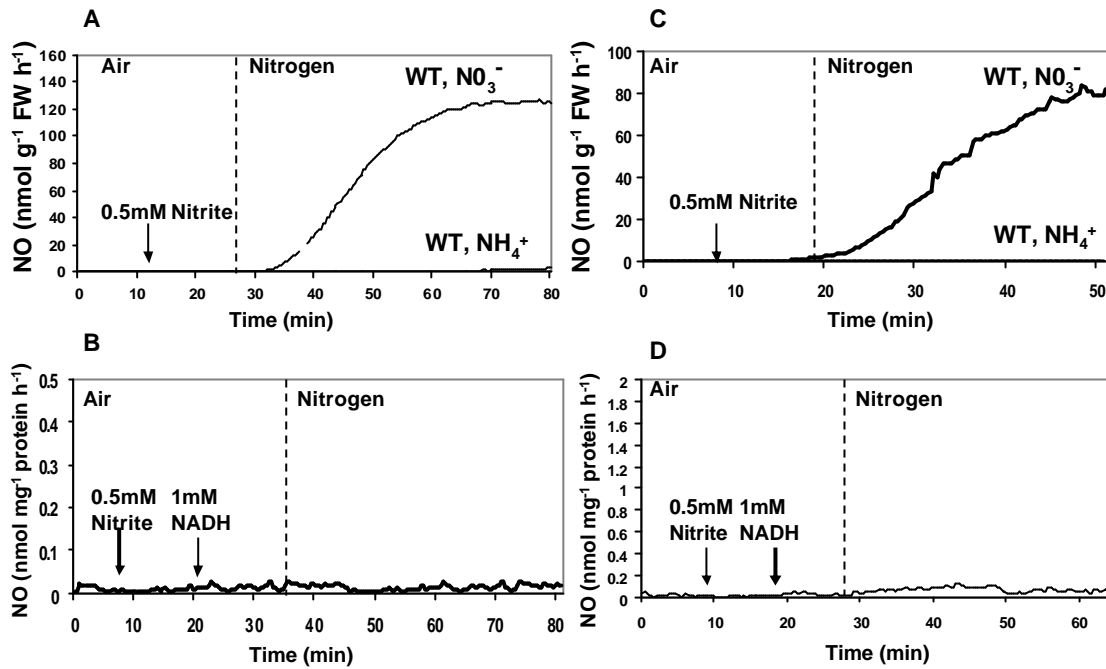


Fig 17: NO-emission from barley (A) or pea (C) leaf slices or isolated leaf mitochondria of barley (B) and pea (D). WT plants were grown hydroponically on nitrate (= with NR activity); or on ammonium plus tungstate (= without NR activity). Mean oxygen uptake for barley leaf mitochondria was 4.5 ± 0.6 ($n=3$) $\mu\text{moles mg}^{-1} \text{protein h}^{-1}$ and oxygen uptake of pea leaf mitochondria was 5.5 ± 0.8 ($n=3$) $\mu\text{moles mg}^{-1} \text{protein h}^{-1}$. Curves are recordings from typical experiments out of 2 to 3.

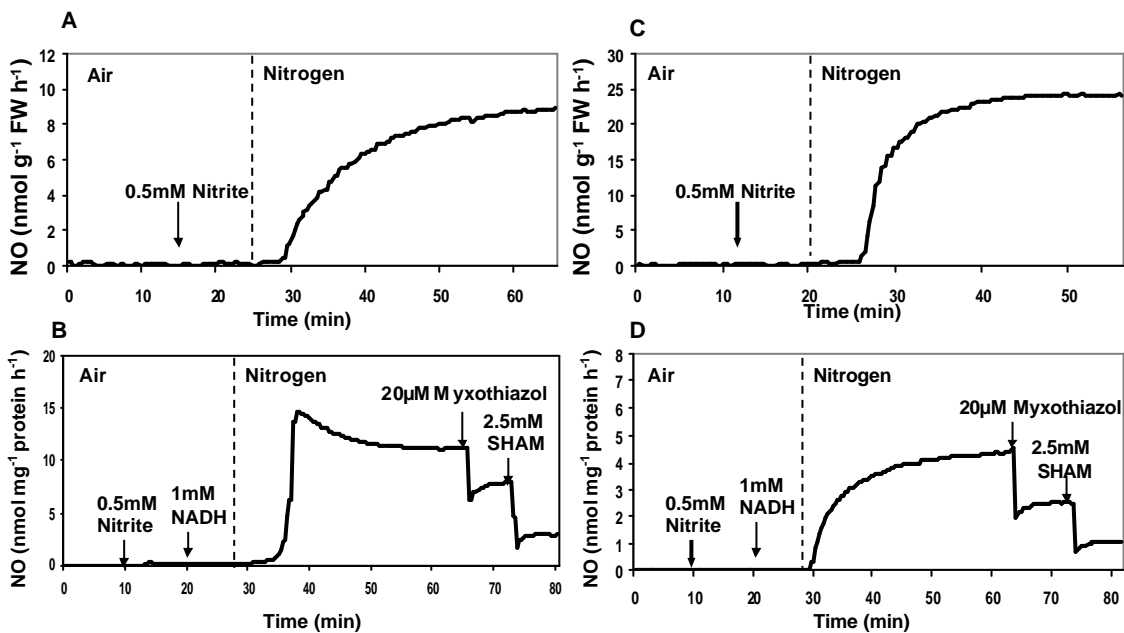


Fig 18: NO emission from root segments of barley (A) and pea (C), and from purified mitochondria of barley roots (B) and pea (D) roots (both grown on ammonium as N-

source). Inhibitors were added as indicated. The very rapid initial drop of the NO emission after inhibitor addition is an artefact of the injection. Curves are representative recordings out of 2 to 3 separate preparations. Mean oxygen uptake for barley root mitochondria was 6.2 ± 0.8 (n=3) $\mu\text{moles mg}^{-1} \text{protein h}^{-1}$ and oxygen uptake of pea root mitochondria 6.6 ± 1.2 (n=3). Curves are recordings from 2 to 3 typical experiments

The above results indicate that leaf mitochondria either lack a nitrite reductase-like activity, or they might have a very strong capacity to scavenge NO. In order to test for the latter possibility, we carried out the following experiment: Mitochondria were isolated from barley leaves and roots. First, both preparations were tested separately for their ability to reduce nitrite to NO. Expectedly, NO emission by root mitochondria was 15 ($\text{nmol mg}^{-1} \text{protein h}^{-1}$), and by leaf mitochondria it was zero. In a 1:1 mixture of root and leaf mitochondria, it was 7.5 (not shown). Thus, there was no scavenging by leaf mitochondria of NO produced by root mitochondria.

From the above experiments it is obvious that mitochondria from roots are able to reduce nitrite to NO, whereas leaf mitochondria produce hardly any NO. It was therefore a question whether mitochondria from other plant species would respond in the same way. Data are summarized in the table.

Table 1: Oxygen uptake from mitochondria isolated from different sources

Source of mitochondria	Oxygen uptake $\mu\text{mol mg}^{-1} \text{ protein h}^{-1}$	NO emission in nitrogen $\text{nmol mg}^{-1} \text{ protein h}^{-1}$
Tobacco root	6.7 ± 0.5	5.5 ± 1
Tobacco leaf	5.5 ± 0.6	0.15 ± 0.02
Pea root	6.8 ± 1.2	5 ± 1
Pea leaf	5.5 ± 0.6	0.05 (one measurement only)
Barley root	6.4 ± 0.4	15 ± 1.5
Barley leaf	4.8 ± 0.7	0.15 ± 0.05
Cauliflower	7.2 ± 0.8	0.23 ± 0.05
Potato tubers	5.8 ± 0.6	0.2 (one measurement only)

6. Nitrite to NO reduction takes place on mitochondrial membranes but not in the matrix:

We have shown that mitochondria from roots or heterotrophic cell suspensions produce NO under anoxia when supplied with nitrite and NADH. NO emission was partly blocked by Myxothiazol (20 μM), a complex ??? inhibitor, and inhibition was strengthened by the AOX inhibitor SHAM (2.5 mM). Mitochondria from leaves were unable to produce NO under the same conditions. Therefore, we examined here whether matrix factors are required for reduction of nitrite to NO. After separation of the mitochondria into membrane and matrix fraction, NO from NADH and nitrite (under nitrogen) was produced exclusively by the membrane fraction (Fig. 19), not by the matrix (not shown). Addition of detergent (0.1 % of Triton-X) to the membrane fraction

completely abolished NO production. In order to find out whether chemiluminescence would detect only part of the NO, we also determined nitrite-dependent NADH oxidation and nitrite consumption by the membrane fraction under nitrogen. Here, the membrane suspensions were preflushed with nitrogen for 15 minutes before addition of NADH and nitrite. Samples were taken at 0 and 60 minutes and nitrite and NADH were measured. While nitrite and NADH consumption roughly matched each other, NO emission was lower, although oxygen was absent (Table 3).

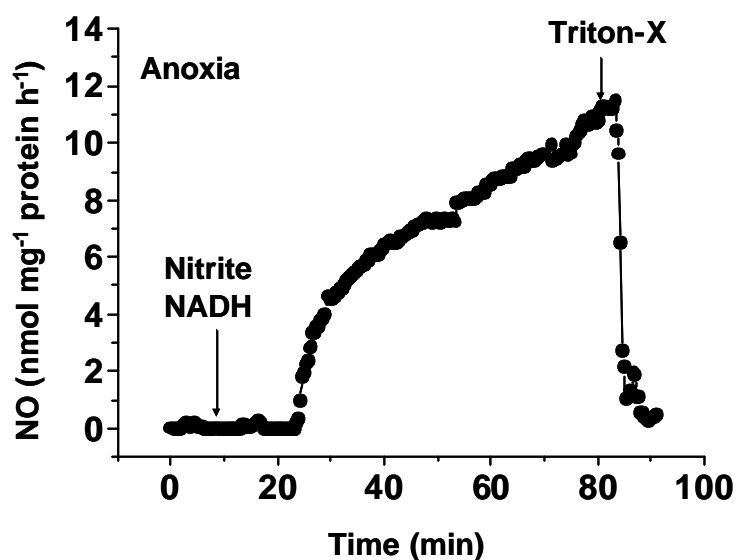


Fig 19: NO emission from purified root mitochondrial membranes after addition of 100 μM nitrite and 1 mM NADH. 4 ml of membrane suspensions (containing approximately 1.2 mg protein) were suspended in buffer as described in material and methods and flushed with nitrogen. 0.1% of Triton-X was injected as indicated in the figure. Data represent one out of 5 independent experiments.

Table 3: Nitrite disappearance, NADH oxidation and NO emission in barley root mitochondrial membranes under anoxia. Rates are $\mu\text{moles mg protein}^{-1} \text{ h}^{-1}$

Nitrite consumption	NADH oxidation	NO emission
11.4 ± 7.4	8.6 ± 3	3.6 ± 1.6

Table 3: Nitrite consumption, NADH oxidation, and NO emission were measured in barley root mitochondria after addition of 100 μM NO_2^- and 100 μM NADH. Results are expressed in nmoles/ml. Results are mean \pm (n=3) and NO emission mean \pm (n=4).

Conclusions:

More work is required in order to find out whether the extremely low aerobic NO emission rates really reflect low NO production due to competition with oxygen at the terminal oxidases, or whether they are due to oxidative scavenging of NO. It should be noted that with purified mitochondria, the hemoglobin cycle cannot contribute to NO oxidation because non-symbiotic

Hb is located in the cytosol. Thus, in mitochondria oxygen/NO competition may seem probable, but the possibility of a quick reaction of NO with ROS cannot be neglected. However, addition of catalase and SOD to purified mitochondria did not improve NO emission (Kaiser, unpublished results). In intact cells, however, the hemoglobin cycle might scavenge NO to the very low aerobic NO emission usually found. Also, it is completely unknown why

leaf mitochondria, most probably having the same terminal oxidases as root mitochondria, are not able to produce NO. The functions of mitochondrial NO are also far from being clear. The

above suggestion that NO may serve to regulate respiratory electron flow through cytoX and AOX is indeed fascinating, but its physiological relevance will depend on the NO concentrations required for inhibition and those that are really reached within mitochondria (which are not yet known). Estimates of in vivo NO concentrations in plants vary widely. While our own estimates of in vivo NO concentrations (tobacco leaves) based on chemiluminescence measurements were in the picomolar or low nanomolar range (Planchet et al.2005), previous studies using other methods gave NO concentrations that were several orders of magnitude higher (0.1–2 μM). Yamasaki *et al.*, (2001) used NO concentrations around 50 nM to cause an inhibition of the steady state membrane potential. The $K_{0.5}$ for cytoX inhibition was also quite high,

approximately 0.1–0.3 μM NO (Millar *et al.*, 2002 and literature cited). Thus, for final conclusions on the possible functions of NO in mitochondria and elsewhere in the cell it is crucial to know the real and potential concentrations of NO in plant tissues, cells, and organelles.

Chapter 2

Nitric Oxide Synthase:

As described in the introduction nitric oxide synthases (NOS) are the primary sources of NO in animals. They are complex, highly regulated enzymes that oxidize arginine to NO and citrulline. Plant NO synthesis, however, appears more complex and may include both nitrite and arginine-dependent mechanisms. The components of the arginine pathway have been elusive as no known orthologues of animal NOS exist in plants. An *Arabidopsis* gene (*AtNOS1*) has been identified that appears needed for NO synthesis *in vivo* and has biochemical properties similar to animal cNOS, yet it has no sequence similarity to any known animal NOS.

There is also ample evidence from biochemical and pharmacological data that an arginine-dependent mechanism analogous to animal NOS reactions exists in plants since 1996. Some of the data are summarized in Table 4

<i>Pisum sativum</i> /Leaves Gaseous NO emission sensitive to NOS inhibitors Haramaty (1996)	Leshem and
<i>Pisum sativum</i> /Leaf peroxisomes Arginine–citrulline assay al. (1999)	Barroso et
<i>Lupinus albus</i> /Roots and nodules Arginine–citrulline assay (1996)	Cueto et al.
<i>Mucuna hassjoo</i> Arginine–citrulline assay Maier (1996)	Ninnemann and
<i>Glycine max</i> / <i>P. syringae</i> -infected cell suspensions al(1998) NO production sensitive to NOS inhibitors oxyhaemoglobin and Arginine–citrulline assay	Delledonne et
<i>Nicotiana tabacum</i> /TMV-infected leaves Arginine–citrulline assay al. (1998)	Durner et
<i>Glycine max</i> /Embryonic axes NADPH–diaphorase activity Puntarulo (1999)	Caro and
<i>Zea mays</i> /Root tips and young leaves Arginine–citrulline assay	Ribeiro et al.

(1999)		
<i>Taxus brevifolia</i> /Callus NO production sensitive to NOS inhibitors		Pedroso et al.
(2000)		
DAF fluorescence		
<i>Nicotiana tabacum</i> /Leaf epidermal cells NO production sensitive to NOS inhibitors		Foissner et al.
(2000)		
<i>Nicotiana tabacum</i> /Cell cultures NO production sensitive to NOS inhibitors		Tun et al.
(2001)		
<i>Arabidopsis</i> /Cell cultures		
<i>Petroselinum crispum</i> /Cell cultures		
<i>Glycine max</i> /Cotyledons Arginine–citrulline assay		Modolo et al.
(2002)		
<i>Arabidopsis</i> Arginine–citrulline assay		Guo et al
(2003)		
<i>Sorghum bicolor L</i> /Seeds NADPH-diaphorase activity		Simontacchi et al.
(2004)		
EPR		
<i>Arabidopsis</i> leaf mitochondria (DAF-FM DA fluorescence)		Guo and
Crawford (2005)		
Different organs of pea by chemiluminescence		Corpas et al
(2006)		
Maize roots by DAF-2DA fluorescence		Zhao et al
a(2007)		
The <i>Cupressus lusitanica</i> var. <i>lusitanica</i> suspension cultures		Zhao et al b
(2007)		
(DAF-DA fluorescence)		
Maize leaves (DAF-2DA fluorescence)		Zhang et al
(2007)		

As shown, most of these data either rely on the use of DAF-fluorescence indicators or on the arginine assay. However, it has been shown recently by the later assay may lead to erroneous results (Tischner *et al*, 2007)

1 NOS activity in crude extracts of roots:

First we determined NOS activity in the crude extracts of barley roots by direct Chemiluminescence. Extracts were desalted twice with Sephadex G-25 columns which eliminated low molecular weight substances (less than 1000 KD). Crude extracts even when supplied with all NOS substrates and cofactors did not produce NO emission. Since the NOS reaction requires oxygen, the measurement was carried out under a stream of air. Under these conditions NO might be oxidized back to nitrite and nitrate. Therefore aliquots of crude extract (50 to 100 μ L) were injected into a hot (95°C) acidic vanadium III mixture as described in material methods. L-Arginine alone caused already significant apparent ($\text{NO}_2^- + \text{NO}_3^-$) production (5 nmoles/h). Next we studied the effect of NOS cofactors and NADPH also had no effect on L-Arginine - mediated $\text{NO}_2^- / \text{NO}_3^-$ production Also incubation of extract with L- Lysine had no effect. Thus, the apparent NOS activity detected as $\text{NO}_2^- + \text{NO}_3^-$ formation had rather untypical properties and may be considered an artifact.

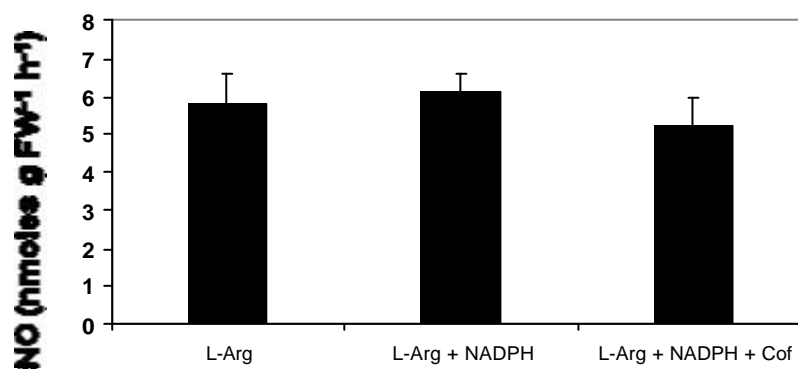


Figure 20: Apparent L-Arginine dependent NOS activity in crude aerobic extracts of barley roots measured as $\text{NO}_2^- + \text{NO}_3^-$ formation as detected by indirect chemiluminescence. 2.5 mM L-Arg, 1 mM NADPH and cofactors (12 μ M BH_4 , 5 μ M Calmodulin, 1.25 mM CaCl_2) were added prior to the samples to activate NOS. Values

are nmoles/mL \pm SD (n= 4). The Initial (nitrite + nitrate) at t=0 was subtracted from each value. Incubation time was 30 minutes.

2 NOS activity in root mitochondria:

The first report on mitochondrial localization of NOS was by Giulivi *et al.*, (1998), who detected NOS activity in purified animal mitochondria, mitochondrial homogenates, and submitochondrial particles, using EPR and oxyhemoglobin to detect NO. Indeed, NOS activity of animal mitochondria appears located in the inner mitochondrial membrane (Ghafourifar and Richter, 1997). Very recent work by Crawford's group indicates that plant AtNOS1, like the animal enzyme, is also located in the mitochondria. This view was based on the following lines of evidence:

- Computational analysis of the NOS1 protein sequence reported a high probability of being targeted to the mitochondria.
- Fluorescence from a p35S-NOS1cDNA-GFP construct strongly overlapped with MitoTracker fluorescence in mitochondria of roots and root hairs examined by confocal microscopy
- NO production in mitochondria isolated from *Arabidopsis* WT and At- NOS1 mutant plants was detected using DAF-fluorescence (Guo and Crawford, 2005).

When root mitochondria were supplied with L-Arg, NADPH, Ca²⁺ calmodulin, and FMN, no NO emission was detected by direct chemiluminescence in air (Fig 21). In order to check for a possible rapid NO oxidation to (nitrite + nitrate), the latter were reduced to NO by acidic V(III) solution, followed by chemiluminescence detection as before. In mitochondrial preparations with all NOS substrates and cofactors, no NO oxidation products could be detected after 30 min (Table 5).

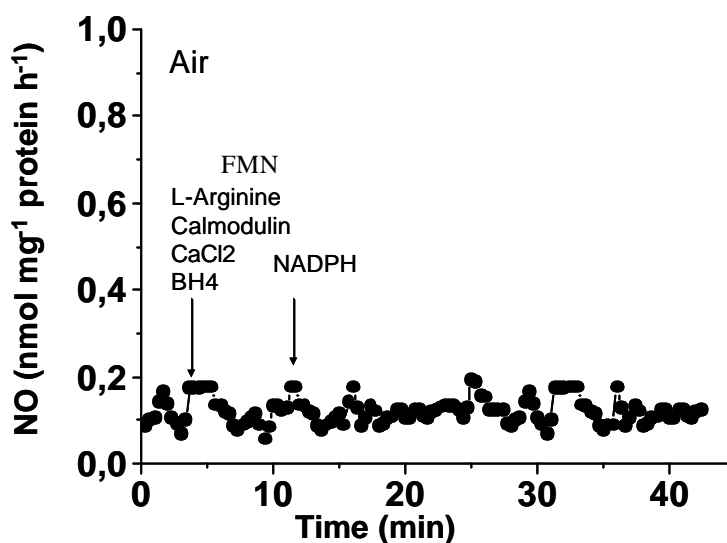


Figure 21: Absence of L-Arginine dependent NO emission from mitochondria as monitored by direct chemiluminescence. The suspension contained 2.5 mM L-arginine (pH 7.4), 10 $\mu\text{g}/\mu\text{l}$ Calmodulin, 1.25 mM CaCl_2 , 12 μM BH_4 , and 1 mM NADPH 10 μM FMN, as indicated. Mitochondrial protein was 15 mg in the total volume of 4 ml.

Table 5: Production of (nitrite+ nitrate) by commercial iNOS (2 U), or by purified mitochondria (mitos, 0.8 mg protein) with substrates and NOS- cofactors in a total volume of 2 mL or mixture of iNOS+mitochondria. If not specifically indicated, all assays contained NADPH (0.5 mM). Values are nmoles/mL \pm SD (n= 4). Initial (nitrite + nitrate) at t=0 was probably due to impurities.

Reaction Mixture	$\text{NO}_2^- + \text{NO}_3^-$	$\text{NO}_2^- + \text{NO}_3^-$
	0 min	30 min
Mitos + L-Arg + Cofactors	5.8 ± 0.4	5.9 ± 0.6
iNOS + L-Arg + Cofactors	1.0 ± 0.3	16.2 ± 1.1

3 DAF-fluorescence from mitochondria:

From the above described experiments it is evident that there is no NOS activity associated with the mitochondria. However Guo and Crawford in 2005 showed NO production in mitochondria isolated from *Arabidopsis* WT and AtNOS1 mutant plants by using DAF-fluorescence. Therefore we also checked for NOS - activity in mitochondria using DAF-fluorescence.

In the presence of DAF-2 is N-nitrosated forming the highly fluorescing triazolofluorescein (DAF-2T). It has been suggested that DAF-2 does not react directly with the NO free radical, but rather with N_2O_3 (Kojma *et al.*, 1998; Planchet and Kaiser, 2006). DAF-2 at constant NO concentration in the reaction medium should give a constant increase (slope) of fluorescence over time, reflecting a constant rate of the formation of the highly fluorescing reaction product, DAF-2T. Changes in the rate of NO production should result in changes in the slope of the fluorescence increase.

Purified root mitochondria (approximately 0.5 mg protein) were incubated with and without L-Arginine, cofactors and NADPH, and fluorescence was followed with time as indicated in the figure.

The mitochondrial preparation by itself produced already a slow increase in DAF fluorescence without L-arginine or any cofactors (Fig 22). Addition of L-arginine (2.5 mM) without other substrates and cofactors increased fluorescence, which is usually interpreted as more rapid NO formation. The NO scavenger cPTIO prevented the fluorescence increase. Unexpectedly, NADPH addition diminished the L-arginine-dependent fluorescence increase instead of stimulating it. NADPH might increase ROS

production by the mitochondria. This, in turn might react with NO and prevent the fluorescence increase. Therefore mitochondria were incubated with SOD and fluorescence in the presence of NADPH was again measured. There was not stimulation of fluorescence increase by presence of SOD (not shown in figure).

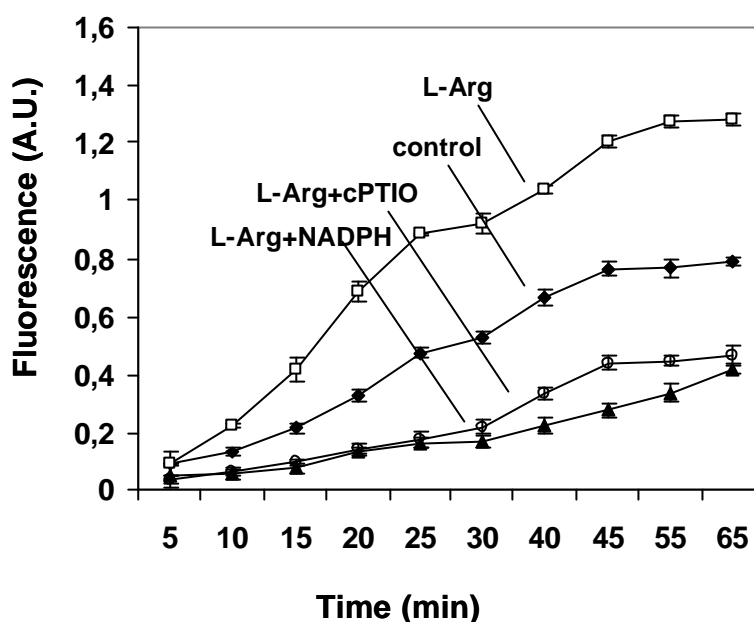


Figure 22: NOS activity in mitochondria as indicated by DAF fluorescence. Mitochondria were incubated for 30 min in the presence of DAF-2 10 μ M. 500 μ M cPTIO, or 1 mM NADPH, or 2 mM L-Arg were added as indicated in the figure. Fluorescence is given as arbitrary units. (means \pm SD, n=4)

Effect of NOS inhibitors on DAF fluorescence:

In order to check whether the observed fluorescence increase is indeed related to NOS, two commonly used L-arginine analogues, L-NAME, (NG-nitro-L-Arg-methyl ester) and L-NIL (L-N⁶-(1-Iminoethyl)-Lysine, acetate) were incubated along with L-arginine. The L-arginine stimulated DAF fluorescence was insensitive to L-NAME and L-NIL added twice at 2.5 the concentration of L-Arginine suggesting that the observed DAF fluorescence was not directly related to a reaction catalyzed by NOS (Fig 23).

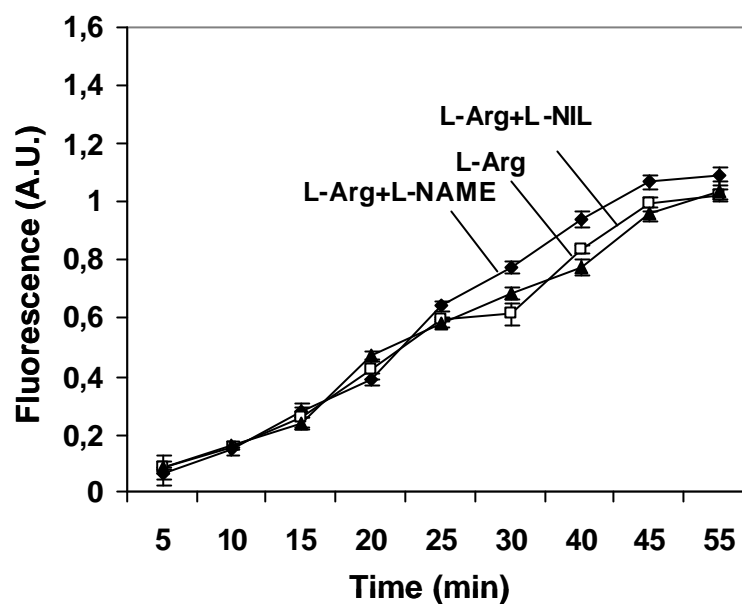


Figure 23: Effect of NOS inhibitors on L-Arginine dependent DAF fluorescence. 2 mM L-Arg and NOS inhibitors (5 mM L-NAME or 5 mM L-NIL) were incubated with mitochondria as indicated in the figure. (means \pm SD, n=4)

DAR-4M is another, more recently developed cell-permeable NO fluorescent indicator with a detection limit for NO of \sim 10 nM. Like DAF-2DA, DAR-4M is trapped inside the cell by the action of esterases. It reacts with NO, in the presence of O₂, resulting in a triazolo-rhodamine analog (DAR-4M T) that exhibits about 40-fold greater fluorescence quantum efficiency than DAR-4M (Kojma *et al.*, 2000) DAR-4M was successfully applied to bioimaging of NO produced by plants (Tun *et al.*, 2006).

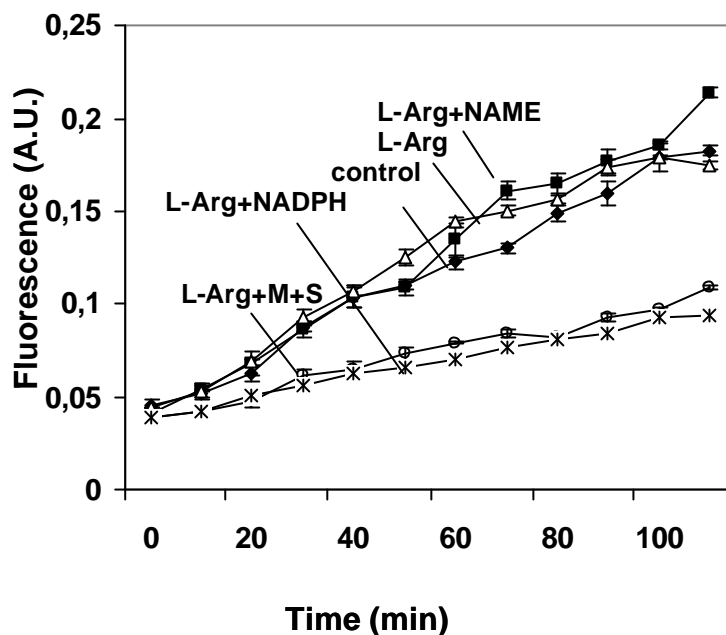


Figure 24: DAR-4M fluorescence with purified mitochondria membranes after incubation with 2.5 mM L-Arg or L-Arg + 1 mM NADPH or 5 mM L-NAME or inhibitors of mitochondrial electron transport (Myxothiazol 20 μ M plus SHAM 2.5 mM). Values are (means \pm SD, n=4).

With DAR-4-M, mitochondrial membranes without any substrate again produced a basic fluorescence increase, which was not increased further with L-arginine, and which was also insensitive to L-NAME, but slightly lower with added substrates. To test whether the increase in the DAF fluorescence was related to the mitochondrial electron transport chain, mitochondria were incubated with Myxothiazol (complex III inhibitor) plus SHAM (inhibitor of AOX) (Fig 24). The combination of both inhibitors prevented fluorescence, suggesting involvement of the electron transport chain in yet unknown way of reaction.

In order to check whether the observed fluorescence was related to membrane or matrix, mitochondria were fractionated into membrane and matrix and checked for the fluorescence in both components by using DAF-2. With purified mitochondrial membranes, the pattern of DAF-fluorescence and its response to substrates, cofactors

and inhibitors was similar as with whole mitochondria, except that L-arginine caused only slight increase in fluorescence.

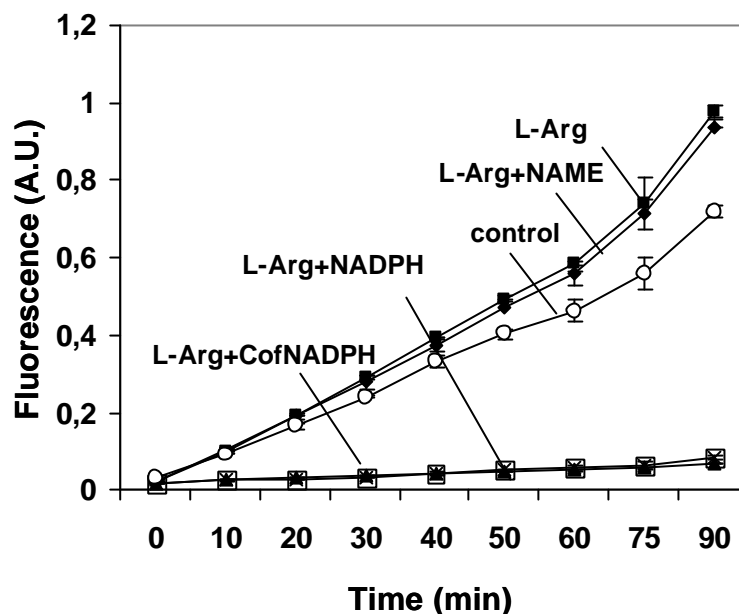


Figure 25: DAF-2 fluorescence from purified mitochondrial membranes preincubated with 2.5 mM L-Arg or L-Arg plus L-NAME or L-Arg + NADPH alone or plus cofactors (12 μ M BH₄, 5 μ M Calmodulin, 1.25 mM CaCl₂, 10 μ M). Values give the fluorescence (A.U.) in different time points. (means \pm SD, n=4)

4 Measurement of commercial recombinant NOS by chemiluminescence:

For comparison, the activity of a commercially available recombinant iNOS (mouse) was also measured by direct and indirect chemiluminescence. According to the manufacturers information, 1 U of iNOS of iNOS supplied with 1 mM L-Arg, 1 mM MgCl₂, 0.1 mM NADPH, 12 μ M BH₄ corresponds to about 60 nmoles NO h⁻¹ at 37°C. Under our conditions only 3.8 nmoles (NO) h⁻¹, or about 6% of the expected activity were released from the solution at 22°C (Fig 24). The NOS inhibitor L-NAME (5 mM)

suppressed 50% of the NO emission, which was reversed by 10 mM L-Arg, demonstrating the well known competitive inhibition by substrate analogs. As expected, NO emission by iNOS was oxygen dependent, being completely suppressed under nitrogen (Fig 24). The observed NO emission is far less than manufacturer description, therefore we measured. In traces of nitrite and nitrate as described before. By this method it become clear that 3 times more (nitrite + nitrate) were formed than NO (Table 6), or in other words, about 70% of the NO formed by iNOS appeared oxidized during the 30 min reaction.

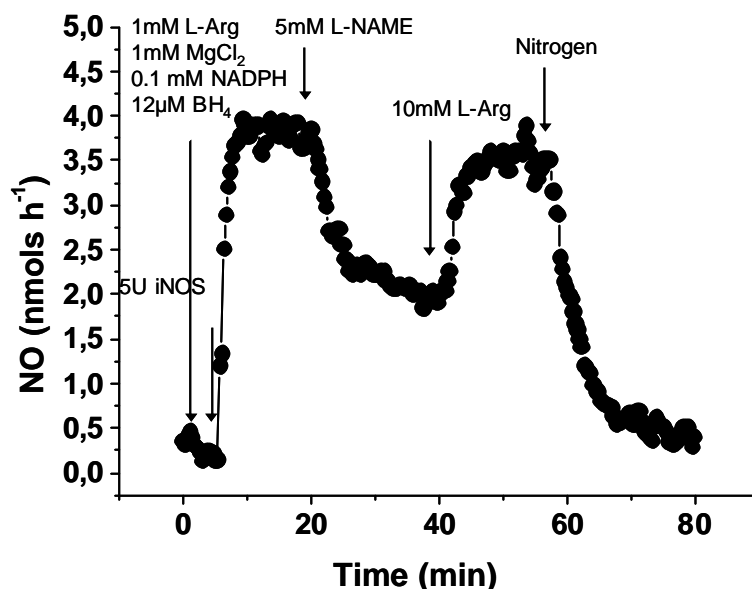


Figure 24: NO generation by purified iNOS (mouse, recombinant) as measured by chemiluminescence. 2 mL of buffer solution (50 mM HEPES pH 7.6) was incubated in a 5 mL glass beaker in head space cuvette at 24°C in air, under vigorous shaking. Substrates, cofactors, were added as shown in the figure.

Table 6: Production of (nitrite + nitrate) by purified iNOS (2 U), with substrates and NOS- cofactors as in Fig.2, in a total volume of 2 ml. If not specifically indicated, all

assays contained NADPH (0.5 mM). Values are nmoles/mL \pm SD (n= 4). Initial (nitrite + nitrate) at t=0 was due to impurities.

iNOS + L-Arg + Cofactors	0 min	30 min
24°C	1.0 \pm 0.3	16.2 \pm 1.1
37 °C	1.2 \pm 0.4	32 \pm 0.8

NO emission from iNOS at optimal conditions: The observed NO by direct and indirect chemiluminescence was still less than indicated by the manufacturer. Therefore NO emission from a solution of iNOS was measured at 37 °C. At this condition NO emission from direct chemiluminescence was 8 nmoles (not shown in the figure) and NO emission by indirect chemiluminescence was 32 nmoles which is double compared to NO emission at 24°C (Table 6).

According to the stoichiometry of the reaction (Stuehr *et al.*, 1991), NADPH consumption should be 1.5 fold the rate of NO formation. Here (Table 6), the rate of NADPH consumption was about 40 nmoles/h, corresponding to 27 nmoles/h of NO formed. The sum of the rate of NO emission by direct chemiluminescence (Fig 24) plus (nitrate + nitrite) formation (Table 5) was 22,4 nmoles/h, which comes close to the rate derived from NADPH oxidation. . Thus, direct + indirect chemiluminescence together appear reliable indicators of NOS activity *in vitro*.

Table 7 : NADPH oxidation by purified iNOS (1U/mL) in presence or absence of substrate L-Arginine and all cofactors. NADPH concentration (nmoles/mL, \pm SD, n= 4) was measured at time zero and after 30 minutes.

Time	NADPH (- L-Arg)	NADPH (+ L-Arg) + Cofactors
0	98.07 \pm 0.7	98.7 \pm 0.62
30	94.4 \pm 3.1	58.05 \pm 10.56

For comparison, we also measured NO-production by iNOS with DAF-2-fluorescence (Fig 25). Upon iNOS addition to the complete reaction mixture, DAF fluorescence increased with time for up to 60 min and then remained constant.

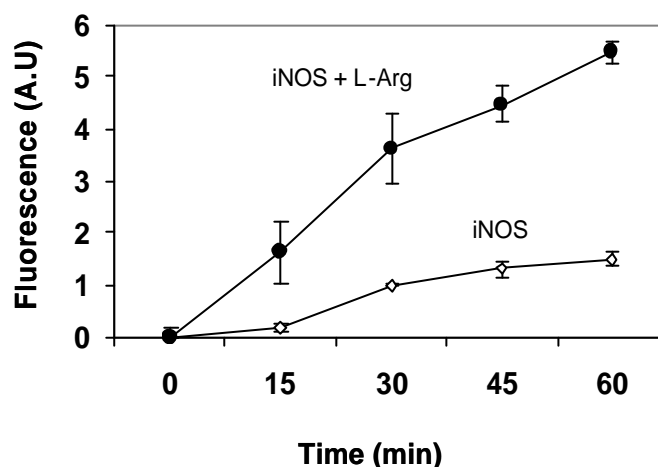


Figure 25: NO formation by commercial iNOS as detected by DAF-2 fluorescence. 2.5 U of iNOS were added at time 0.

5 Scavenging of NO by the mitochondria using NOS as a source of NO:

As shown above, an iNOS assay resulted in a direct release of 3.8 nmoles NO into air and gave 15.4 nmoles of (nitrate + nitrite) in 30 min (Table). While a suspension of mitochondria was emitting NO from nitrite and NADH under anoxia, the rate of nitrite-dependent NO production detected by chemiluminescence was lower than consumption

of NADH and nitrite (compare Table 3). In air, no NO emission was detectable and thus, all NO might have been oxidized, which would also prevent direct detection of NOS activity by chemiluminescence. For nitrite-dependent NO production, determination of NO oxidation to (nitrite + nitrate) was not possible because of the high nitrite background. In order to check whether mitochondria had NOS activity but would quantitatively oxidize the NO produced in air, a mitochondrial preparation was supplied with L-Arg plus NADPH and cofactors (Table 4). However, no NO was detected, neither directly nor as (nitrate plus nitrite), confirming complete absence of NOS activity in root mitochondria.

Due to the lipophilicity of NO, the NO concentrations in the lipid phase will be much higher than in the water phase, which might favour aerobic NO oxidation by mitochondria. Therefore, as an additional check for mitochondrial oxidation of NO, we used iNOS in mixture with mitochondrial membranes. NO emission from iNOS itself (without mitochondria added) was 4 nmoles h^{-1} . Here, the V(III) method gave 15.2 nmoles of (nitrate + nitrite) (Table 4). When mitochondrial membranes were injected into a sample of iNOS, direct NO emission was decreased by almost $2/3$ (Fig 26), yet the amount of (nitrite + nitrate) was only slightly increased (by indirect chemiluminescence).

Therefore we measured the NOS activity in closed and open cuvette. In a closed cuvette nitrite+nitrate formation from purified NOS and also mixture of NOS+mitochondria were much higher than in the open cuvette suggesting that mitochondria do scavenge NO and the NO rapidly oxidized back to volatile compounds like N_2O_3 . Therefore in closed cuvette Nitrite + Nitrate formation was increased (not shown)

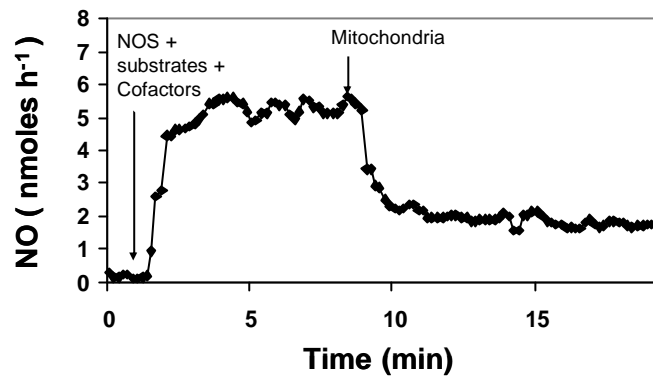


Figure 26: Scavenging of NOS- derived NO by root mitochondria. NO emission was measured by chemiluminescence. After steady state was reached, 1ml of mitochondria (approximately 0.8 mg protein) was injected as indicated.

Conclusions

Neither desalted root extracts nor purified mitochondria from roots contained a significant (constitutive) NOS-like activity, but appeared to produce NO only from nitrite. Anoxic reduction of nitrite to NO by root mitochondria required the electron transport chain, but no matrix components. NO produced by iNOS was partly oxidized back to nitrite (and nitrate). This NO oxidation was not accelerated by mitochondria, although direct NO emission by iNOS was lowered. It seems possible that mitochondria oxidized NO to a gaseous intermediate like N_2O_3 , which would largely escape from the solution together with NO. Nevertheless, if mitochondria would produce NO in air, at least some should be detectable as NO emission. However, this was not the case, confirming our previous conclusion that mitochondrial reduction of nitrite to NO is inhibited in air. Unfortunately, DAF and its derivatives, which are supposed to react with N_2O_3 , behaved not as reliable NO indicators because of their unexpected response to NOS substrates, inhibitors and cofactors.

Chapter 3:

Role of nitrate and NO in plant/pathogen interaction:

Introduction

In incompatible plant–pathogen interactions, induction of defence involves signalling pathways leading ultimately to the manifestation of localized cell death, which is termed 'hypersensitive response' (HR). Reactive oxygen species and reactive nitrogen species, primarily nitric oxide appear to be part of signalling pathways leading to the HR (Delledonne *et al.* 1998; Bolwell 1999; Durner & Klessig 1999; Clarke *et al.* 2000; Klessig *et al.* 2000; Neill *et al.* 2002; Lamotte *et al.* 2004; Zeier *et al.*, 2004). Recently, the expression of a bacterial NO dioxygenase in transgenic plants to reduce NO levels has demonstrated the role of NO in the induction of cell death, the activation of the phenylpropanoid pathway, and initiation of the generation of reactive oxygen species (ROS; Zeier *et al.*, 2004). NO and ROS may rapidly react with each other to form highly toxic peroxynitrite, and they may be directly or indirectly (as peroxynitrite) involved in destroying both, cells of the pathogen and of the host, at least in high concentrations. Rises in plant NO production or NO synthase (NOS) activity after pathogen contact have been detected in various incompatible interactions, for instance after inoculation of soybean, *Arabidopsis* and tobacco with different avirulent strains of *Pseudomonas syringae* (Delledonne *et al.*, 1998; Clarke *et al.*, 2000; Conrath *et al.*, 2004; Mur *et al.*, 2006), after infection of tobacco with tobacco mosaic virus (TMV), or during the response of pepper to an incompatible isolate of *Phytophthora capsici* (Durner *et al.*, 1998; Requena *et al.*, 2005). Previous work has suggested that in plants, as in animals, a nitric-oxide-synthase NOS-like reaction should mediate NO production during the HR. But the existence of AtNOS1 has been questioned recently (Zemojtel *et al.*, 2006, Crawford *et al.*, 2006). Some reports suggested NR to play a role in

production of NO during the HR (Yamamoto-Katou *et al.*, 2003, 2006). Therefore the role of NR derived NO was assessed in tobacco plants in response to avirulent *Pseudomonas syringae*.

Pathogen-elicited NO production has been implicated as a critical component in the initiation of the hypersensitive cell death response (HR) (Delledonne *et al.*, 1998; Clarke *et al.*, 2000; Pedroso *et al.*, 2000), and synergistic interactions between NO and ROS are likely to play a key role during this process (Delledonne *et al.*, 2001; Zeier *et al.*, 2004a). Independently from ROS, NO has been shown to positively regulate the production of salicylic acid (SA), up-regulation of the SA-dependent pathogenesis-related protein 1 (*PR-1*), and induction of further defence genes like *PAL* (phenylalanine ammonia lyase), *PAD4* (phytoalexin-deficient 4), *GST1* (glutathione-S-transferase 1), and *AOX1* (alternative oxidase 1) (Durner *et al.*, 1998; Delledonne *et al.*, 1998; Huang *et al.*, 2002a; Zeier *et al.*, 2004a). In addition, phytoalexin accumulation in potato and soybean has been demonstrated to be mediated by NO (Noritake *et al.*, 1996; Modolo *et al.*, 2002). An involvement of NO in non-host and basal resistance was implicated more recently. The non-host HR of tobacco induced by *P. syringae* pv. *phaseolicola* (*Psp*) is thought to be preceded by NO formation, and cell death is delayed by the NOS inhibitor L-NAME or the NO scavenger cPTIO (Mur *et al.*, 2006). Moreover, bacterial lipopolysaccharides, typical cell surface components of gram-negative bacteria, provoke NO formation, enhance NOS activity, and elicit defence gene induction in *Arabidopsis* (Zeidler *et al.*, 2004). As these responses are attenuated in the putative *Arabidopsis* NO synthase mutant *Atnos1* (Guo *et al.*, 2003), and *Atnos1* plants are more susceptible towards virulent *P. syringae*. Considering the lack of a plant NOS-like enzyme, it is astonishing that effects caused by inhibitors of mammalian NO synthases are still taken as evidence for an involvement of NO in plant processes. NOS inhibitors predominantly

involve L-arginine derivatives, which in principle are able to block any L-Arg-dependent event in plants (Planchet and Kaiser, 2006).

1 Cryptogein induced cell death is independent of presence or absence of NR.

Recently our group has demonstrated (Planchet *et al.*, 2006) that when cryptogein (10 nM) was infiltrated into tobacco leaves from nitrate grown-plants (attached to the plant or detached with the petiole in nutrient solution), lesions became visible after 20–24 h. Here those experiments from Planchet *et al.*, 2006 were repeated. We examined whether NR was required for the cryptogein induced cell death. When leaves from plants totally devoid of NR activity, such as leaves from ammonium-grown plants, or from wild-type (WT) plants that had been cultivated on a medium containing 500 μ M sodium tungstate instead of molybdate, cryptogein-induced lesions developed normally. Only in the *nia30* mutant, lesion formation was somewhat slower than with WT leaves, but still clearly visible (Fig 27)



Figure 27: Lesion development after 24 hours in tobacco leaves (WT and antisense NiR transformant (271) upon infiltration with the fungal elicitor (20 nM) cryptogein. Symptoms were more frequent in both ammonium and nitrate grown plants. Figure shows one representative out of 4 independent experiments (Data repeated from Planchet *et al.*, 2006)

2 Is NR is required for hypersensitive response induced by TMV?

When tobacco plants were treated by injection with nitric oxide (NO)-releasing compounds, the sizes of lesions caused by tobacco mosaic virus (TMV) on the treated leaves and on upper nontreated leaves were significantly reduced (Song and Goodman, 2001). One conclusion was that reduction in TMV lesion size was caused by NO released from the NO donors. Since evidence has been accumulating that NOS like activity does not exist in plants, we studied whether NR dependent NO plays a role in the induction of HR in response to TMV. Here we used *Nicotiana rustica* which is best suitable plant for studies with TMV. For plants totally devoid of NR, we used leaves

from ammonium-grown plants and leaves from WT plants that were cultivated on a medium containing 500 μM sodium tungstate instead of molybdate.



Figure 28: Lesion development in tobacco leaves of nitrate/ammonium grown plants 5 days after inoculation with Tobacco Mosaic Virus (Lesions become visible after a temperature shift from 32°C to 24°C (N-Nitrate, A-Ammonium))

When tobacco plants were inoculated with TMV and incubated at temperatures of $>28^{\circ}\text{C}$, the replication and spread of the TMV are not restricted, necrotic lesions are not formed, and PR genes are not induced. However, when the infected plants are moved to lower temperatures (22°C), PR proteins accumulate and the HR is rapidly activated. Therefore after inoculation plants were kept under 32°C for two days and then shifted to 24°C for 2 days. After two days the necrotic regions were observed in both nitrate and interestingly plants, indicating that the HR induced by TMV is independent of presence or absence of NR (Fig 28), as shown previously for the fungal elicitor Cryptogein (Planchet *et al.*, 2006).

3 Is NR required for HR induced by avirulent *Pseudomonas syringae*?

Pseudomonas syringae is a gram-negative bacterium with polar flagella (Agrios, 1997). In the tobacco-*Pseudomonas solanacearum* pathosystem, NO donors themselves were able to provoke a HR (Huang and Knopp, 1998) and to induce cell death in *Arabidopsis* cell suspension cultures when present at concentrations producing NO similar to amounts generated following challenge by avirulent pathogens (Clarke *et al.*, 2000). Soybean and *Arabidopsis* cell suspensions inoculated with *Pseudomonas syringae* produced NO with a pattern similar to H_2O_2 accumulation (Delledonne *et al.*, 1998; Clarke *et al.*, 2000). In this case, an initial rapid, but transient, stimulation of NO accumulation is induced by both avirulent and virulent *Pseudomonas syringae* strains. However, this is followed by sustained production of NO only in the cells inoculated with the avirulent strain. In their study by using EPR, Modolo *et al.* (2005) found nitrite as a major source of NO production by *Arabidopsis thaliana* in response to *Pseudomonas syringae* pv *maculicola*. They also found that mitochondrial electron transport was responsible for nitrite dependent NO production during the interaction. But in our case we could not find any nitrite dependent NO production from leaf mitochondria. The reason for this discrepancy is not clear so far.

To further investigate the role of NR dependent NO during the incompatible interaction different tobacco mutants (WT, NiR deficient line 271, nia mutant) were grown on either 5 mM NO_3^- or 5 mM NH_4Cl for one week and checked for NR activity. NO production from WT, or 271 leaves was 2.4 ± 0.4 and 22 ± 3 respectively. NO emission was negligible in all the lines grown on ammonium (Table 8).

Table: NO emission from tobacco leaves (*Nicotiana tabacum* cv Gatersleben or of NR deficient nia double mutant or NiR deficient clone '271') under light (air) grown for one

week on 5mM nitrate (for induction of NR) or 5mM ammonium (for suppression of NR activity). (nd-not detected)

Plant type	NO (nmol g ⁻¹ FW h ⁻¹)
WT NO ₃	2.4 ± 0.4
WT NH ₄ ⁺	nd
271 NO ₃	22 ± 3
271 NH ₄ ⁺	nd
Nia NH ₄ ⁺	nd

When WT and 271 tobacco plants (grown on nitrate as a sole source of nitrogen) were challenged with *Psp* at concentrations of 0.01 OD, dry colorless lesions were developed on the site infiltration within 48 hours (Fig 29). These macroscopic lesions are characteristic of the HR. The lesion development was more severe in 271 leaves than in WT leaf which may indicate a correlation with NO production. (Table 8).

In contrast to nitrate-grown plants, in ammonium grown plants there was no HR visible even after 4 days. This may indicate that the HR is indeed NO-dependent, contrary to the situation with Cryptogein or TMV. However, other reasons seem also possible which will be examined below.

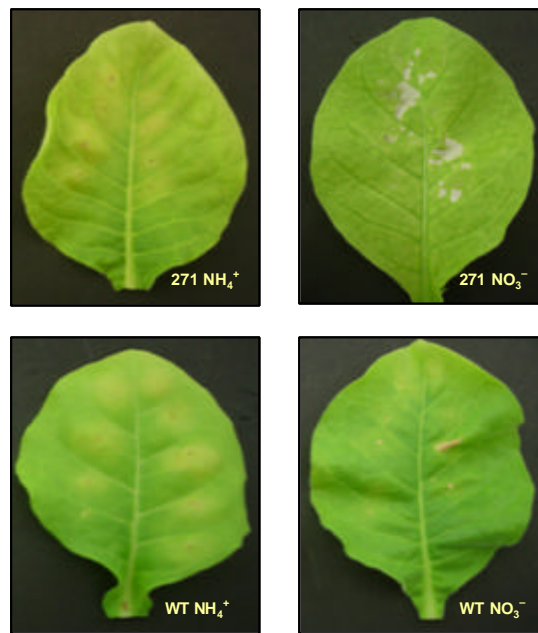


Figure 29: Lesion development after 24 hours in tobacco leaves (WT and antisense NiR transformant (271) upon infiltration with avirulent *Pseudomonas syringae* pv *phaseolicola*. Symptoms were more frequent and severe in 271 than in WT (grown on nitrate). HR formation was strongly delayed in plants grown on ammonium. Figure shows one representative result out of 5 independent experiments.

4 Is *in vivo* bacterial growth correlated with lesion formation?

The HR is a mechanism used by plants, to prevent the spread of infection by microbial pathogens. HR is characterized by the rapid death of cells in the region surrounding the infection. In a way HR is analogous to the innate immunity system found in animals and commonly precedes a slower systemic response, which ultimately leads to systemic acquired resistance (SAR). To test this hypothesis we quantified the bacterial spread in the leaves after infiltration of the bacteria. As expected, in WT and 271 plants grown on nitrate there was less bacterial growth when compare to the plants fed with ammonium. In 271 plants grown on ammonium and also in *nia* plants grown on ammonium, bacterial growth was higher than in WT grown on ammonim (Fig 30). Thus bacterial growth was negatively correlated with lesion formation.

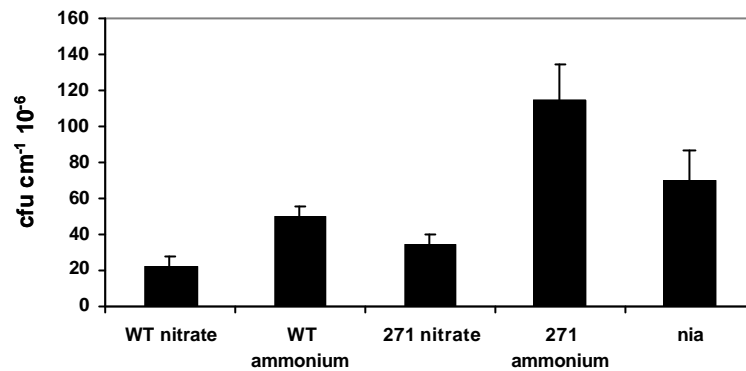


Fig 30: Bacterial growth in different tobacco mutants and different growth conditions. One week before the experiment plants were transferred to nitrate (3 mM) or ammonium (3 mM). Suspensions of *P. syringae pv.phaseolicola* were adjusted to OD 0.01 and then pressure infiltrated in to the leaves. Bacterial growth was monitored after 24 hours ($n = 6 \pm SD$). For further details compare material and methods.

5 Is bacterial growth correlated with sugar or amino acid contents in leaf cells or in the leaf apoplast?

The above result indicate that bacterial growth was favored in ammonium grown plants. There might be two reasons for this observation 1) Less nitric oxide production due to lack of NR expression 2) different growth conditions for the bacteria in these plants under ammonium.

To test the hypothesis we measured the total sugars and amino acids in these plants. Because of bacterial infection may interfere with HPLC analysis, sugars and amino acids were measured in uninoculated leaves.

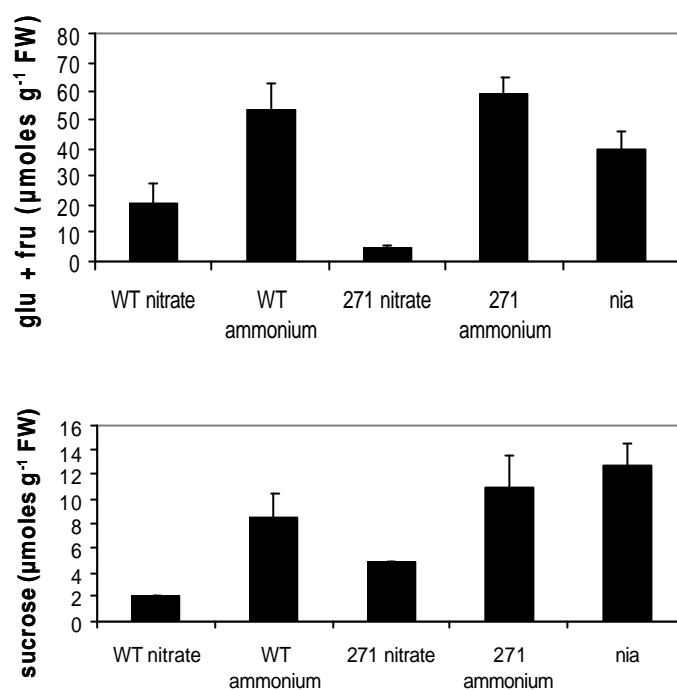


Figure 31: Total Trioses (Glucose+Fructose) and Hexose (Sucrose) levels ($\mu\text{moles g}^{-1}$ FW) in tobacco leaves (WT, NiR and *nia*). Data obtained each from eight leaves from different plants. ($n = 8 \pm \text{SD}$)

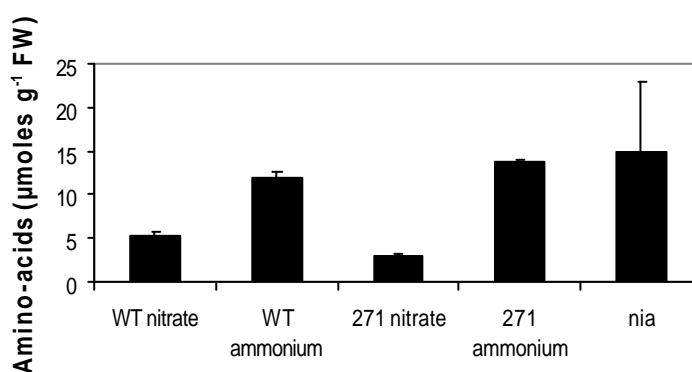


Figure 32: Amino acid levels ($\mu\text{moles g}^{-1}$ FW) in tobacco leaves. (WT, NiR and *nia*). Data obtained from eight leaves of 8 independent plants. ($n = 8 \pm \text{SD}$)

Indeed sugars and amino acids were higher in leaves of plants fed with ammonium (Fig 31 and 32) than on nitrate.

Pseudomonas syringae is a non-invasive, extracellular pathogen which colonizes the host intercellular spaces outside the plant cell wall at least in the early infection phases. Therefore in order to determine the key factors (amino acids and sugars) responsible for initial bacterial growth, apoplastic fluids were extracted from the leaves and measured bacterial growth was measured as before.

Apoplastic sugars (sucrose and fructose + glucose) contents were much higher in ammonium grown plants and followed the same patterns as total sugars (Fig 33). Due to very small volume of samples it was been impossible to measure amino acids.

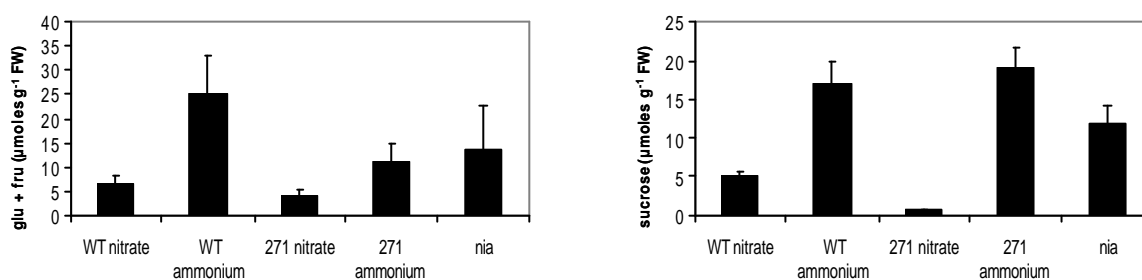


Figure 33: Apoplastic sugar contents in the leaves of tobacco plants. Data obtained from 4 leaves from independent leaves. ($n = 4 \pm SD$). Apoplastic solutions were obtained as described in Material and Methods.

6 To what extent is an HR induced by cryptogin restricting bacterial growth?

As shown above, apoplastic nutrients appeared an important factor restricting bacterial growth in the leaves. However, defense reactions might add to that. To test this in to nia leaves grown on ammonium, bacteria were inoculated together with 20 nm cryptogin

or 10 μ M salicylic acid, and optical density was measured in extracts after 8 hours and 14 hours. However, it was first examined whether cryptogein or SA would by themselves effect the bacterial growth (Fig 34). Clearly, neither compounds had a direct effect.

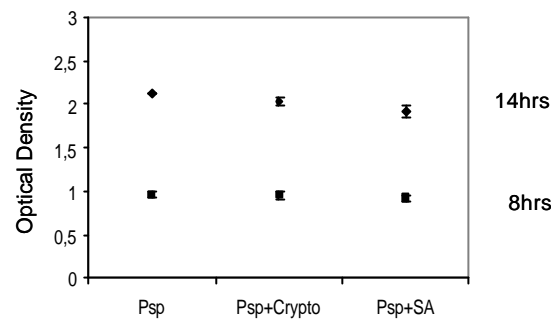


Figure 34: Effect of 20 nM cryptogein or 10 mM Salicylic acid on in vitro Psp growth in ammonium grown *nia* leaves ($n = 4 \pm$ SD).

Next 20 nM cryptogein was coinfiltrated with Bsp on one half of the leaf, and as a control Psp alone was injected in the other half of the leaf. Expectedly cryptogein caused a typical HR. *Psp* alone did not cause any visible HR. Bacterial growth was measured in those leaves in an undamaged area of 1 cm around the lesions. Psp growth was drastically suppressed in the presence of cryptogein. Growth of virulent Pst was also checked under the same condition and was also strongly reduced in the presence of cryptogein.

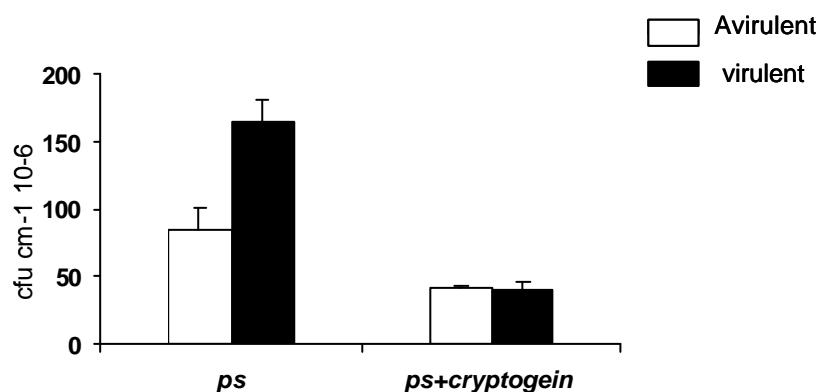


Figure 35: Effect of cryptogein on growth of avirulent and virulent *Pseudomonas* in *nia* plants after 48 hours. 20n M cryptogein was infiltrated along with the bacteria 0.01

concentration ($n = 4 \pm \text{SD}$) *Pseudomonas syringae* pv *phaseolicola* and *Pseudomonas syringae* pv tomato were the avirulent and virulent strains respectively.

The above results demonstrate a heavy restriction of bacterial growth by the HR. In order to determine any NOS like activity besides NR to contribute to the HR a possible NO emission was measured from the ammonium leaves infected with *Psp*. There was no NO production in the light. After switching to the dark NO emission was increased a bit (0.12 ppb) but there was no further increase after 20 hours (Fig 36).

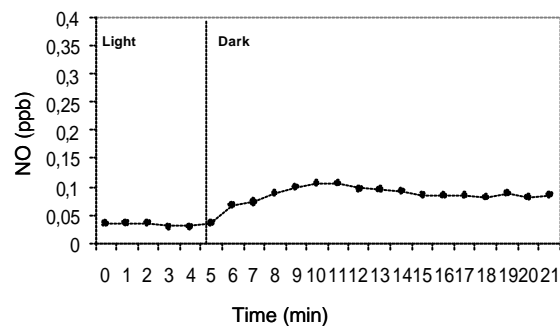


Figure 36: Time course of NO emission from tobacco *nia* leaves after infection with *Psp*. Data represent one representative experiment out of 4 independent measurements.

As pointed out before, direct measurement of NOS by NO emission may be masked by rapid NO oxidation. Therefore we measured (nitrite + nitrate) content in extracts from leaves infiltrated with *Psp*. There was no significant (nitrite + nitrate) indeed in the leaves (not shown).

In leaves from nitrate grown plant the HR rapidly developed, as shown before. In the leaves of ammonium grown plants the HR developed only very slowly. As a final control, leaves inoculated with *Psp* were exposed to NO gas (10 ppm for 24 hours), and PR-expression was followed by northern blotting.

Pathogenesis-related (PR) proteins are a group of plant proteins whose synthesis is induced in response to pathogen infection. Production of PR proteins has been related to

disease resistance and is considered as an indication of a defense response. Figure 37 shows that *Psp* drastically increased the expression of PR-1 in leaves of both nitrate and ammonium grown plants, but induction was strong in leaves of nitrate grown plants as has to be expected in a classical HR.

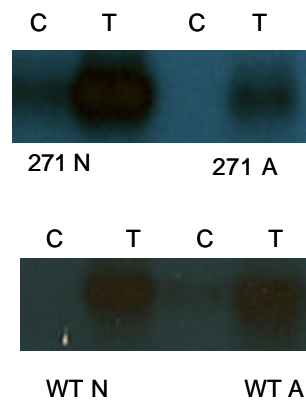


Figure 37: PR-1 gene expression in response to *Pseudomonas syringae pv phaseolicola*. (N-Nitrate A-Ammonium, C-control, T-inoculated) (Northern blots were carried out with kind help from Dr. Joergen Zeier and Dr Tatiana Mishina, Botany II, Wuerzburg).

Pseudomonas syringae pv phaseolicola (OD 0.01) was infiltrated into the leaves of 271 and WT Tobacco leaves and samples are harvested after 24 h post infiltration. Total RNA from mock or *Pseudomonas* treated tobacco leaves was extracted separated by electrophoresis, blotted and hybridized with PR-1a tobacco cDNA probes, as described in Materials and Methods.

Concluding remarks:

There has been increasing evidence that nitric oxide (NO) plays a central role in plant-pathogen interactions. Many studies on the roles of NO focus on its effect in promoting the HR, but knowledge about other factors besides NO which may affect bacterial growth in the host are limited. Infiltration of avirulent *Pseudomonas syringae p.v. phaseolicola* into tobacco leaves of nitrite-reductase antisense transformants (which produces high nitric oxide levels) leads to a more rapid and severe HR compared to wild type. Similarly, growing plants on ammonium as the sole N-source, which prevents NO synthesis, strongly delayed lesion formation. Weak and slow lesion formation was expectedly correlated with high bacterial growth. This might point to the classical role of NO in the HR. However, apoplastic sugar and amino acid levels were much higher in plants grown on ammonium compared to nitrate-grown plants. Thus, levels of organic nutrients in the host leaf apoplast might also affect bacterial growth. Indeed, preliminary data indicate that flushing ammonium-grown leaves with NO does not affect bacterial growth.

C Discussion:

1 NO production by root mitochondria

It was a major task of this work to analyse the function of mitochondria as a source of NO in higher plants. In plant organs that actively reduce nitrate, the product of the reaction nitrite, reaches only low concentrations. However, under hypoxia or anoxia, nitrite to ammonia reduction is impaired, and as a consequence nitrite accumulation takes place (Botrel *et al.*, 1996). Under such conditions, nitrite appears partly reduced to NO, by NR itself but also by plant mitochondria.

Root tissues, as well as mitochondria isolated from roots, were obviously able to reduce nitrite to NO via mitochondrial electrontransport. This also indicates that nitrite at the concentrations used was able to enter cells and mitochondria *in situ*. The apparent NO production rate was extremely low in air, and much higher under hypoxia/anoxia. This behaviour is in marked contrast to that of purified NR, where nitrite to NO reduction was only slightly increased by anoxia (Planchet *et al.*, 2005). NO scavenging by respiring mitochondria was not sufficient to explain the large difference in aerobic and anoxic NO emission. The I_{50} was in the range of 0.05% oxygen, corresponding to an oxygen concentration in water of about 0.63 μM , at a nitrite concentration of 500 μM . Thus, without any further investigation of the reaction kinetics it appears that the oxygen affinity of the reaction site is several orders of magnitude higher than the affinity for nitrite.

The sensitivity of NO production to the complex III inhibitor, Myxothiazol, and to the AOX inhibitor, SHAM, suggests that both terminal oxidases (CytOx and AOX) may participate in NO production, in confirmation of previous data obtained with a green alga (Tischner *et al.*, 2004) or mitochondria purified from tobacco suspension cells

(Planchet *et al.*, 2005). Whether an additional nitrite-NO reductase protein is involved, or whether this activity is inherent to the terminal oxidases themselves is not clear yet.

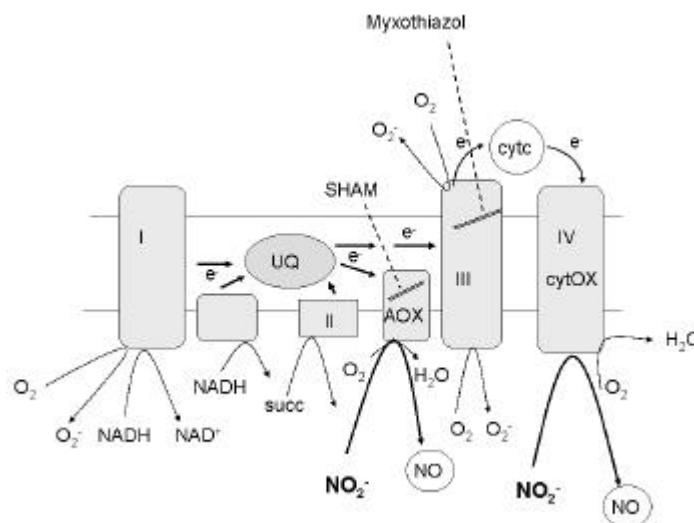


Figure 38: Reduction of nitrite to NO by the electron transport chain of plant mitochondria. For reasons of simplicity, only one NADH dehydrogenase is shown in the diagram. According to the inhibition sites of Myxothiazol (complex III) and salicylhydroxamic acid (AOX), both terminal oxidases appear to possess nitrite:NO reducing activity, which would also explain a possible competition of nitrite and O_2 , leading to a very low NO formation rate in air. It is also shown that superoxide (O_2^-) can be produced at several different sites and in close neighborhood to NO, which would facilitate formation of oxidized NO species. \hat{U} inhibition, *UQ* ubiquinone, *succ* succinate, *cyt c* cytochrome c, *AOX* alternative oxidase, *Cyt ox* cytochrome oxidase

2 Scavenging of NO by mitochondria:

As shown in the results section after injecting 186.2 pmol NO into the pure water nearly 95% of the added NO was recovered. This indicates that NO is rather stable in water. But in the more complex mitochondrial buffer approximately 40% of NO was scavenged under the stream of air and 30% under the stream of nitrogen suggesting that

components of the buffer already caused some quenching. Mitochondria suspension alone quenched 83% in air and 75% in nitrogen. Interestingly, mitochondria supplemented with 1mM substrate (NADH) scavenged approximately 25% more than mitochondria without substrate. 1 mM NADH alone and caused no scavenging. One possible explanation could be a generation of a more superoxide as in the presence of substrate. This superoxide would rapidly react with NO, as pointed out.

In another set of experiments we checked the effect of changing concentration of mitochondria on NO quenching. Both under air or nitrogen it was observed that with increasing concentration of mitochondria the rate of NO consumption increased.

Another approach

With root mitochondria, nitrite-dependent NO production could be detected only under anoxia. There may be several reasons: Either, oxygen competes efficiently with nitrite at the terminal oxidase, as originally suggested. Or, NO is formed, yet rapidly oxidized thereby escaping detection by gas phase chemiluminescence. The problem is difficult to solve, since it is not possible to measure traces of nitrite+ nitrate (pmoles/mL) formed on the large background of added nitrite (100 nmoles/mL). It is also not possible to measure nitrite consumption, because nitrite could be 'recycled' through NO oxidation; and it is also not possible to measure NADH consumption, because in air NADH is much more rapidly consumed by respiratory electron flow to oxygen.

To tackle the problem, we have used iNOS as NO source in air, and checked for the effect of mitochondria on this NO emission. Addition of mitochondria to iNOS decreased direct NO emission, but without increasing (nitrite + nitrate) formation from NO. In closed vessels not flushed with air, (nitrite+ nitrate) formation was higher than in open vessels (not shown) under continuous shaking, which indicates that NO or oxidized gaseous intermediates like NO₂ or N₂O₃ may have been formed. If

mitochondria accelerate the formation of the gaseous intermediates which escape together with NO in the chemiluminescence measurement, this might explain the above discrepancy between the decrease in NO emission without increase in the formation of (nitrite + nitrate).

3 Why do leaf mitochondria not reduce nitrite to NO?

According to the above data and to literature, a mitochondrial 'nitrite:NO reductase activity' now appears to exist in bacteria, green algae, higher plants, and animals (Kozlov *et al.*, 1999; Tischner *et al.*, 2004; Planchet *et al.*, 2005). Considering the fact that this activity covers such a wide range of organisms including photoautotrophic algal cells, it is even more surprising that leaf mitochondria were not able to produce NO from nitrite. Whether that is due to a different property of leaf and root terminal oxidases, or to the absence from leaf mitochondria of an additional nitrite:NO reductase protein associated with the oxidases (see above), is not yet known. However, an absence of NO emission from leaf mitochondria is certainly not due to a specifically high scavenging capacity, as shown.

An obvious question then, is, why do root mitochondria make NO but leaf mitochondria do not. Anoxic mammalian mitochondria also produced NO from nitrite (Kozlov *et al.*, 1999) in a Myxothiazol-sensitive reaction. As mammalian mitochondria synthesize NO primarily via NOS (Giulivi, 1998), the reduction of nitrite to NO was considered secondary and was suggested to serve for a recycling of NO oxidation products (Kozlov *et al.*, 1999). This cannot be the purpose of nitrite to NO reduction in plants, where nitrite is a normal intermediate of N-metabolism.

Roots, in contrast to leaves, may frequently experience oxygen deficiency in water-logged soils. Under those conditions, nitrate and nitrite may serve as alternative electron

acceptors and may partly replace fermentation for NADH reoxidation during glycolysis, even though electron flow through NR was comparatively low (Stoimenova *et al.*, 2003). However, the above-described rates for the reduction of nitrite to NO are probably too low to be relevant as an electron sink for the maintenance of a minimum energy metabolism.

The lack of ability of leaf mitochondria to produce NO might somehow be related to photosynthesis. However, nitrite-dependent NO production was also lacking in mitochondria from other non-green tissues like potato tubers and cauliflower inflorescences (Table 1). On the other hand, as mentioned above, mitochondria from non-green tobacco cell suspensions reduced nitrite to NO, although with a lower capacity than root mitochondria (on a protein basis). Thus, more work is required to find out what the molecular basis for the observed differences is and which physiological purpose they might fulfil.

4 Possible roles of Nitrite-NO reduction by roots and root mitochondria

A specific property of roots with respect to nitrite-dependent NO production was also suggested by the detection of Stöhr and colleagues (Stöhr *et al.*, 2001; Stöhr and Ullrich, 2002; Meyer and Stöhr, 2004), of a nitrite:NO reductase associated with the PM-bound NR in roots. Thus roots, in contrast to leaves, would even have two separate membrane-bound systems to reduce nitrite to NO. Such redundancy suggests that nitrite-dependent NO formation in roots serves a specific and important purpose. As a secondary messenger, NO appears to trigger cell death in plant–pathogen interactions (Delledonne *et al.*, 1998; Wendehenne *et al.*, 2001, 2004) or in differentiating xylem elements (Gabaldon *et al.*, 2005). In roots and stems of flooding-tolerant plants, hypoxia leads to

the differentiation of air-conducting 'aerenchyma', which may involve the induction of lysigenous cell death. Ethylene biosynthesis also triggers aerenchyma formation. However, the conversion of ACC into ethylene requires oxygen and thus does not work under anoxia. On the other hand, NO production is optimal under anoxia, and thus NO might take over some of the signalling for lysigenous cell death when oxygen is rapidly and completely consumed. While this is speculative at this point, the fact that root and leaf mitochondria are different suggests at least some connection of NO production with specific aspects of root metabolism in relation to hypoxic/anoxic conditions.

Another interesting question is whether reduction of nitrite to NO by mitochondrial electron transport might provide an alternative to fermentation under anoxia. Ethanol and lactic acid are the major end products of fermentation in roots, and both are toxic if they accumulate to high concentrations. Reduction of nitrate to nitrite by cytosolic NR, and further reduction of nitrite to NO by NR or by mitochondria, might represent an alternative to fermentative NAD⁺ regeneration. Indeed, we could show that roots expressing NR, which accumulate nitrite under anoxia and emit NO, produce much less ethanol and lactate and acidify their cytosol less than NR-deficient roots, which do not form nitrite and, therefore, emit no NO (Stoimenova *et al.*, 2003). In collaboration with the Robert Hill group we demonstrated that mitochondria readily oxidized NADPH and NADH in anoxia via externally facing dehydrogenases, using nitrite as an electron acceptor and forming NO, resulting in the production of ATP (Stoimenova *et al.*, 2007) data not shown here. Anaerobic ATP production and NAD(P)H rates were higher in rice than in barley. This suggests that mitochondrial operation under anaerobic conditions may be more sustainable in the hypoxia-resistant plant (rice) than in the hypoxia-sensitive plant (barley). This mitochondrial anaerobic process may contribute to the oxidation of reduced pyridine nucleotides formed in the cytosol during glycolysis and in

lipid breakdown, but it may also contribute to the oxidation of the intramitochondrial substrates, particularly in rice. NO is, at least, one of the products of nitrite reduction.

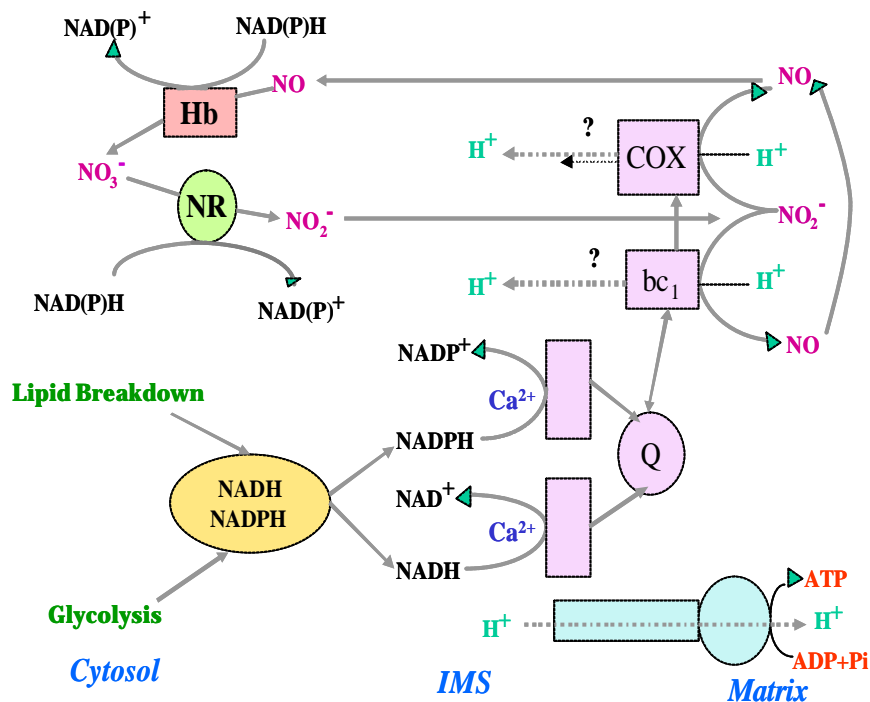


Fig 39: Operation of plant mitochondria under hypoxic conditions. Glycolytic fermentation and lipid breakdown in hypoxia result in the increase of cytosolic NADH and NADPH. Externally facing Ca²⁺-dependent mitochondrial dehydrogenases oxidize NADH and NADPH and transfer electrons to ubiquinone (Q). At levels of oxygen below saturation of cytochrome *c* oxidase (COX), nitrite can serve as an alternative electron acceptor at the sites of complex III and COX. Nitric oxide (NO) formed in this reaction is converted by hypoxically induced hemoglobin (Hb) to nitrate (NO₃⁻). The latter is reduced to nitrite (NO₂⁻) by hypoxically induced nitrate reductase (NR). ATP is synthesized due to proton pumping possibly at the sites of complex III (bc₁) and COX. IMS intermembrane space of mitochondria (Figure from Stoimenova *et al.*, 2007)

5 NOS or no NOS?

The most commonly used NOS-activity measurement is based on the conversion of radiolabelled L-arginine to L-citrulline. The test has been recently questioned (Brooks 2004) since components of urea cycle can also perform this conversion without NO synthesis and are also sensitive to NOS inhibitors (Reisser, 2002). Our chemiluminescence system could directly detect NO emission from commercial iNOS, and 70 to 75% of the NO appeared oxidized to nitrite/nitrate. The sum of NO+ (nitrite + nitrate) formed by iNOS was within the rate range derived from NADPH oxidation. We could not detect any NO formation or (nitrite + nitrate) formation from mitochondria in air, using L-arginine, NADPH and all NOS cofactors. Thus, it is obvious that root mitochondria have no NOS activity. With desalted root extracts supplied with all NOS cofactors and substrates, we also found no direct NO emission. However, a low (nitrite + nitrate) formation was detected, which was responsive to L-Arg, but not to NADPH and NOS cofactors. Thus, as for mitochondria + DAF-2, this (nitrite + nitrate) formation of crude root extracts appeared not as a typical NOS activity.

6 Is DAF a reliable NO indicator?

There is evidence that DAF-2 does not react directly with the NO free radical, but rather with oxidized forms e.g. N_2O_3 (nitrous anhydride) (Kojima *et al.*, 1998, Espey *et al.*, 2001) which may be formed via NO_2 even in the absence of oxygen (Lim *et al.*, 2005), and which in aqueous solution should finally end up as nitrite. Here, we also observed DAF fluorescence from mitochondria. While this fluorescence was partly scavenged by cPTIO, some rather unusual observations suggest the fluorescence increase to be an artefact. These were mainly i) insensitivity to NOS inhibitors and ii) a negative response to NOS substrates. At this state, the

mechanism behind the DAF-fluorescence increase observed with mitochondria remains unclear.

7 Do nitrate and NR play a role in the HR?

In previous work, we have shown that NO emission from leaves, roots, cell suspensions or enzyme solutions into the gas phase can be quantified by chemiluminescence, which is highly sensitive and specific (Planchet *et al.*, 2005). Accordingly, the method should be suited to quantify NO emission also during the HR. An early, transient 'NO burst' has been considered as a signal for the induction of cell death in the HR. We detected only very low NO in leaves from ammonium grown plants, which did not respond (increase) to *Pseudomonas* treatment. NOS seemed not involved in PCD, this prompted us initially to expect that NR might be involved in NO signalling. The interest in NR as a potential source for NO was further motivated by recent findings that an early cryptogei-induced event was a nitrate efflux (Wendehenne *et al.*, 2002), and that NR was induced by pathogen signals (Yamamoto *et al.*, 2003) Previous results from our lab shown that the hypersensitive response (HR) of tobacco triggered by the fungal elicitor cryptogei occurred independent of the presence or absence of nitrate reductase (NR). One conclusion was that NR-dependent NO formation plays no role in the HR. The same trend was obvious when tobacco leaves Infected with TMV. Vast numbers of studies into the roles of NO focus on its effect in promoting the HR, but knowledge about other factors besides NO which may affect bacterial growth in the host are limited. In tobacco leaves of clone 271, which have a continuous and drastic overproduction of NO especially in the light, infiltration of avirulent *Pseudomonas syringae p.v. phaseolicola* lead to a more rapid and severe HR compared to wild type. Similarly, growing plants on ammonium as the sole N-source, which prevents NO synthesis, delayed lesion

formation. Weak and slow lesion formation was expectedly correlated with high bacterial growth. This might point to the classical role of NO in the HR. However, apoplastic sugar and amino acid levels, which were much higher in plants grown on ammonium compared to nitrate-grown plants. Thus, levels of organic nutrients in the host leaf apoplast might also affect bacterial growth. Indeed, preliminary data indicate that flushing ammonium-grown leaves with NO does not affect bacterial growth. At the same time, PR-1-expression was induced in both systems, indicating a typical HR, and bacterial growth was reduced in the presence of cryptogein. Thus interdependence of bacterial growth, NO production and HR is complex and not unifactoral

D Material and methods:

1 Plant materials:

Wild-type (WT) plants, *nia* 30 double mutant, and clone 271 of *Nicotiana tabacum* cv. Gatersleben were cultivated in a vermiculite/sand mixture (2 parts vermiculite/1 part sand) for 2–3 weeks and from then on hydroponically for a further 3–4 weeks. Plants of growth stage 5–7 weeks that were selected for similar size for the experiments. During the sand/vermiculite phase plants were watered with full-strength nutrient solution twice a week.

Barley (*Hordeum vulgare*. L cv *Cameron*) seeds were surface sterilized with 0.1% H₂O₂, washed four times with distilled water and then grown on hydroponically in plastic pots for 2 to 3 weeks in growth chambers with artificial illumination (HQI 400W, Schreder, Winterbach, Germany) at 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (PAR), a day length of 16 hours and day/night temperatures of 25°C/20°C. The full strength nutrient solution (8L per 40 plants) for 'nitrate' grown plants contained: 5 mM KNO₃, 1 mM CaCl₂, 1 mM MgSO₄, 0.025 mM NaFe-EDTA, 1 mM K₂HPO₄, 2 mM KH₂PO₄, 1 mM K₂SO₄ and trace elements according to (Johnson et al. 1957) respectively. The root medium was flushed continuously with pre-moisturized air. Fresh nutrient solution was provided every other day. During the first four days on hydroponics, plants were gradually adapted (1/10, 1/4, 1/2, 3/4 and of full-strength) to full-strength nutrient solution. Experiments were performed with roots harvested 6h into the light phase.

2. Preparation of root segments and leaf slices

2.1 Root segments:

After a quick rinse with deionized water, root systems were carefully spread on a glass plate and gently blotted with tissue paper. One gram of the lowest 2–3 cm of the secondary and tertiary roots (including the root tips) were set aside, cut into 5 mm segments and submerged in a glass vessel containing 10 ml 20 mM HEPES-KOH, pH 7.0, 5 mM KNO₃ (or 5 mM NH₄Cl, respectively) and 50 mM sucrose. Sucrose was added to make sure that no carbohydrate starvation took place.

2.2 Leaf slices:

Freshly harvested leaves were double rinsed with deionized water and cut into 1 cm long and 1–3 mm wide segments, avoiding the mid-rib portion. The leaf segments (1 g FW) were vacuum infiltrated for 2–3 min in 10 ml 25 mM HEPES-KOH pH 7.4 and 0.5 mM CaSO₄. After infiltration, segments were washed once and subsequently suspended in the same buffer for the NO measurements.

3 *Nicotiana tabacum* cell suspension cultures

Linsmaier & Skoog medium (LS medium)

Macro-elements

NH ₄ NO ₃	20.61 mM
KNO ₃	18.79 mM
MgSO ₄ · 7H ₂ O	1.50 mM
KH ₂ PO ₄	1.25 mM
CaCl ₂ · 2H ₂ O	2.99 mM

Micro-elements

Na ₂ EDTA, 2H ₂ O	0.1 mM
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$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 mM
H_3BO_3	0.1 mM
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.1 mM
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	29.91 μM
KI	5.0 μM
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	1.03 μM
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.10 μM
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.11 μM

Vitamins

Myo-inositol	100.0 mg L ⁻¹
Thiamine HCL	0.40 mg L ⁻¹

Hormones

Auxin	0.22 mg L ⁻¹
Cytokinin	0.18 mg L ⁻¹
Sucrose	30 g L ⁻¹

The cell suspension derived from tobacco (*Nicotiana tabacum* cv. *Xanthi*) was cultured in 300 mL Erlenmeyer flasks containing 100 mL of LS medium pH 5.8 (Linsmaier and Skoog, 1965) at a constant temperature of 24°C and a continuous illumination (15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR), on a rotary shaker (New Brunswick Scientific, N.J., USA) at 100 rpm. Subcultures were made weekly by transferring 20 mL of the cell suspension into 80 mL of fresh growth medium. Three days after subculturing cells were used for the experiments.

Ammonium cell suspension cultures totally devoid of nitrate were grown on LS medium with small modifications: 1.5 mM NH_4Cl instead of NH_4NO_3 and KNO_3 , and addition of 2.35 mM MES in order to maintain the pH around pH 5.5. Tungstate cell suspension cultures were similar to the nitrate cells or to the ammonium cells, but $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ was replaced by tungstate (150 μM)

4 Mitochondria preparation:

The isolation procedure followed previously published methods (Nishimura et al. 1982; Vanlerberghe et al. 1998) with slight modifications. All procedures were carried out at 4°C. Roots (25 to 30g fresh weight) and tissues were homogenized with an Ultra Turrax (IKA, Janke and Kunkel, Germany) in 250ml of homogenization medium containing 0.3 M sucrose, 100 mM HEPES pH 7.6, 0.1% (w/v) fat free milk powder, 0.6% PVP (w/v), 1 mM EDTA, 2 mM MgCl₂, 4 mM Cysteine, 5 mM KH₂PO₄, and a ready-to-use protease cocktail ('Complete', Roche, Mannheim, Germany, 1 tablet in 100 ml medium). The homogenate was filtered through 8 layers of cheesecloth. The filtered cell homogenate was centrifuged at 3000g for 15 minutes, and the supernatant was centrifuged again at 10 000g for 20 minutes. The resulting pellet was suspended in 4 ml of medium containing 20 mM HEPES pH 7.6, 0.3 M sucrose, 2 mM MgCl₂, 1 mM EDTA, and 0.5 mM KH₂PO₄. The mitochondria were further purified on a discontinuous Percoll gradient composed of the following steps (bottom to top): 3 ml of 60% (v/v), 4 ml of 45% (v/v), 4 ml of 28%(v/v), 4 ml of 5% (v/v), all containing 250 mM sucrose, 20 mM HEPES pH 7.6 and 0.1% defatted milk powder. The mitochondria (4 ml) were layered on top, and the gradient was centrifuged at 30,000g for 30 minutes. The mitochondrial fraction appeared at the interface between the 45 and 28% (v/v) layer and was collected, diluted in 8 ml suspension medium, centrifuged (17 000g, 10 min), and resuspended twice in 8 ml, in order to remove Percoll. The final mitochondrial pellet was suspended in 8 ml medium.

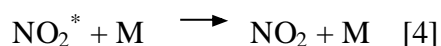
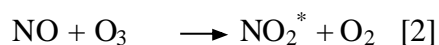
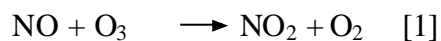
4.1 Fractionation of mitochondria: Mitochondria were fractionated into membrane and matrix according to (Millar *et al.*, 2001). Approximately 1.5 mg of mitochondrial protein was incubated in 1 ml of 20 mM HEPES (pH 7.4), freeze-thawed in liquid

nitrogen three times and then centrifuged for 25 minutes at 20,000g. The supernatant was representing the soluble

Components of the matrix, and the pellet were retained as the total membrane fraction. Each fraction was diluted 1to3 fold for NO measurements and also for fluorescence measurements.

5 Gas Phase NO measurements:

The detection of NO was performed by a NO analyzer (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland). The principle for measuring NO is the measurement of light emission resulting from the reaction of nitric oxide (NO) with ozone (O_3). NO is measured directly, NO_2 indirectly. The reactions between NO and ozone can be described by the following formulae:



NO_2^* : the excited nitrogen dioxide molecule

M: deactivating colliding partners (N_2 , O_2 , H_2O)

The method is based on a first order chemical reaction between NO and an excess amount of ozone (O_3) [1]. A significant fraction of nitrogen dioxide (NO_2) produced in the reaction is in an excited state NO_2^* [2]. The spontaneous deactivation of NO_2^* occurs with emission of light [3] where each molecule of NO_2^* emits one photon. By far the

larger fraction of NO_2^* loses its excitation energy without light emission by colliding with other molecules.

5.1 Description of the analyzer CLD 770 AL ppt

The figure 39 shows the components of the analyzer. Despite the fact that the CLD 770 AL ppt analyzer contains two reactions chambers (pre- and main chamber), the instrumentation is in principle a one channel analyzer. In the small pre-chamber, NO reacts completely with ozone. All other reactive plant volatiles are carried out to the main reaction chamber where they react also under light emission. The measuring (NO) signal is the difference of two measurements: One without prechamber reaction, and one with it. Each reaction is usually followed by counting the photons emitted during 10 sec. Thus, one measuring point was usually collected every 20 sec. The pre-chamber serves also to determine the interference signal (pre-reaction) and the chemical zero point. The actual chemiluminescence reaction that produces the measurement signal takes place in the main reaction chamber. In order to maximize sensitivity, the reaction of NO with O_3 needs to take place under low pressure. A powerful, external vacuum pump generates a reaction chamber vacuum of approximately 15 mbar. The low pressure provided by a vacuum pump is driving both the gas sample and the ozone into the chamber. The photomultiplier collects the light directly and not through a mirror. It converts and amplifies the emitted light impulses into current pulses. To increase sensitivity, the PMT is thermoelectrically cooled to -10°C . The inside of PMT housing is flushed with dry air in order to prevent condensation. Ozone is generated from molecular oxygen (dry at 99 %) by a high-voltage electrostatic ozone generator in large excess. A microprocessor calculates the NO signal in ppb. A customer made software

based on Visual Designer (PCI-20901SS, Ver. 4.0, Tuscon, Arizona, USA) was used to process the data

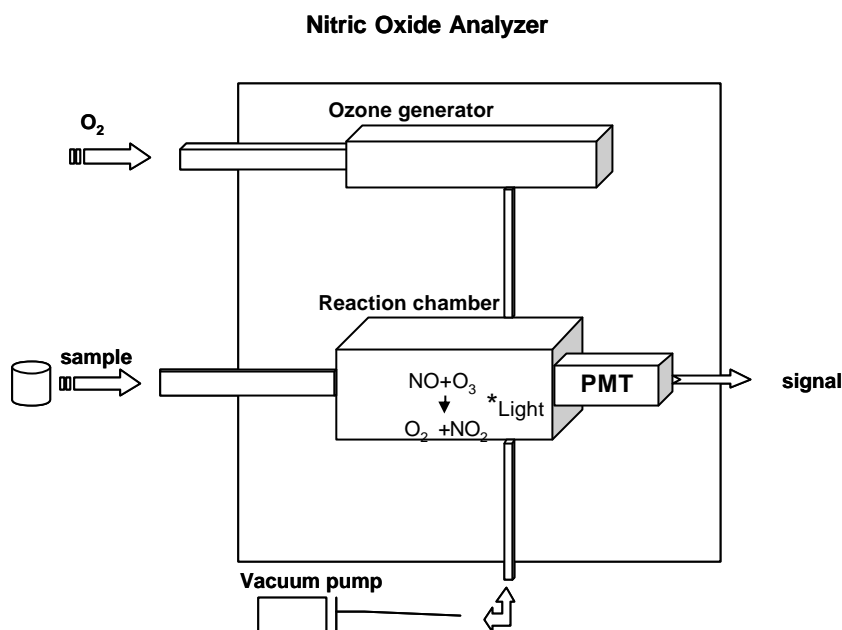


Fig 39: Assembly of nitric oxide analyzer based on the principle of ozone-mediated chemiluminescence. NO from gaseous samples reacts with ozone under formation of electrical excited nitrogen dioxide. Emitted light (chemiluminescence) is detected by a photomultiplier tube (PMT).

5.2 NO gas measurements

For experiments with detached leaves, the leaves were cut off from the plant and immediately placed in nutrient solution, where the petiole was cut off a second time below the solution surface. The leaves (petiole in nutrient solution) were placed in a transparent lid chamber with 2 or 4 L air volume, depending on leaf size and number. In the case of mitochondrial suspensions or enzyme solutions, a defined volume was placed in a Petri-dish in a transparent cuvette (1 L) mounted on a shaker or on a magnetic stirrer, depending on the experiments. A constant flow of measuring gas

(purified air or nitrogen) of 1.5 L/min was pulled through the chamber and subsequently through the chemiluminescence detector by a vacuum pump connected to an ozone destroyer. The measuring gas was made NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Whether the experiment needed of a purified air devoid of CO₂, the obtained NO-free gas stream was immediately conducted afterwards in a column containing sodium lime. Calibration was routinely carried out with NO free air (0 ppt NO) and with various concentrations of NO (1 to 35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. Light was provided by a 400 W HQI-lamp (Schreder, Winterbach, Germany) above the cuvette. Quantum flux density could be adjusted within limits (150-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) by changing the distance between lamp and cuvette. Air temperature in the cuvette was continuously monitored, and was usually about 20°C in the dark and 23 to 25°C in the light.

6 NO scavenging by mitochondria:

To check scavenging of NO by mitochondria, a solution containing a defined amount of NO was prepared by flushing 10 ml distilled water with gas containing 95 ppm NO in nitrogen. According to the solubility of NO in water at atmospheric pressure (1.9 $\mu\text{mol ml}^{-1}$), the NO concentration in the solution was 180 pmol ml^{-1} . Aliquots (2 ml) of this NO solution were injected into 10 ml of water, mitochondrial buffer alone or buffer with mitochondria (1.5–2.5 mg protein in 10 ml buffer). All solutions were stirred in a head space cuvette flushed continuously with purified air or nitrogen, as indicated. Immediately after injection the NO starts to escape from the stirred solution into the gas phase, where its concentration was continuously measured for 20 min until NO emission

came to an end. Integration of the emission curve revealed the total amount of NO recovered. In order to determine whether NO scavenging by mitochondria (in air or nitrogen) would be different under respiratory or non-respiratory conditions, scavenging was also compared with or without the addition of 1 mM NADH.

7 DAF- and DAR-4M- fluorescence:

For fluorometric NO determination, the fluorophore 4,5-diaminofluorescein, (DAF-2), the cell-permeable diacetate (DAF-2DA), or, occasionally, diaminorhodamine-4M (DAR-4M) was used (Alexis Biochemicals, Gruenberg, Germany). Mitochondrial suspensions, membranes or pure enzyme solutions were preincubated with and without L-arginine and various cofactors and NOS inhibitors for 20 minutes in ice. Then 10 μ M DAF-2 was added to each aliquot and DAF-2T fluorescence was measured using a spectrofluorometer (Jasco FP-6500) with 495 nm excitation and 515 nm emission wavelength (3nm band width). For DAR-4M fluorescence, 560 nm excitation and 575 nm emission were used. Fluorescence was expressed as arbitrary fluorescence units (AU), and was measured at the same instrument settings in all experiments.

8 Nitric Oxide synthase (NOS) assay: Measurement of NOS activity was performed in duplicate for each sample (root extract or mitochondria) in a reaction medium containing 1 mM LArg, 0.1 mM NADPH, 12 μ M tetrahydrobiopterin, 1 mM MgCl₂, 10 μ g/ml calmodulin, 1.25 mM CaCl₂ 10 μ M FMN, and mitochondria or mitochondrial fractions, or root extracts or purified NOS, as indicated. The mixture was continuously shaken (125 rpm) in the head space cuvette and NO emission was measured by chemiluminescence.

9 Nitrate/nitrite trace analysis (“indirect chemiluminescence”): To check for traces of nitrate/nitrite produced by a potential NO oxidation, 100 to 500 μ l of the respective samples were injected into a reducing reaction mixture (50 mM Vanadium (III) chloride in 1M HCl) at 90°C, under continuous stirring. After the reaction chamber, the gas was passed through 100 ml of 1M KOH to protect the analyzer from HCl carried over. The production of NO was calculated by subtracting the 0 time value which represents non enzymatic NO production from (suspension medium and cell components) (Braman and Hendrix, 1989). An assay with commercial recombinant iNOS (mouse) was performed as a positive control. This iNOS assay contained 50 mM HEPES (pH 7.4), 1 to 5 U of NOS, 1 mM L-Arg, 1 mM MgCl₂ 0.1 mM NADPH, 12 μ M tetrahydrobiopterin, 10 μ M FMN.

10 Nitrite consumption: Separate aliquots of the mitochondrial suspensions were rapidly mixed with a reaction mixture containing: 600 μ l sulphanilamide (1%), 600 μ l of N-(1-naphthyl)- ethylenediaminedihydrochloride (0.02 %), and 300 μ l of zinc acetate (0.5 M). After 25 min of incubation at 24 °C, the mixture was cleared by centrifugation (16 000 g, 5 min), and the nitrite content from the supernatant was determined photometrically (Hageman and Reed, 1980)

11 Measurement of NADH and NADPH oxidation: NADH or NADPH oxidation by mitochondria was allowed to proceed for 60 minutes and stopped with 0.2M KOH. The NADH or NADPH content of samples at time 0 and after 60 min was measured spectrophotometrically at 340 nm after centrifugation of the samples at 15,000 g for 10 min

12 Growth of plant pathogens and infection:

12.1 Growth and treatment of *Pseudomonas* strains: *Pseudomonas syringae* pv. *phaseolicola* were grown at 28°C in King's B medium containing the appropriate antibiotics (Zeier *et al.*, 2004). Overnight log phase cultures were washed three times with 10 mM MgCl₂ and diluted to a final concentration of OD 0.02, OD 0.005 or OD 0.002. The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1 ml syringe with a needle. Control inoculations were performed with 10 mM MgCl₂. Bacterial growth was assessed by homogenising disks originating from infiltrated areas of 3 different leaves in 1 ml 10 mM MgCl₂, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28 °C for two days.

12.2 Cryptogein treatment:

Young leaves, weighing about 2 g per leaf, were selected from four to eight week old tobacco plants. Leaves were directly infiltrated with cryptogein through the abaxial epidermal layer with a 2 mL plastic syringe without needle. The cryptogein was prepared from the stock solution by suitable dilution with buffer (5 mM HEPES-KOH, pH 7.0). Control leaves were treated in the same way with buffer only. The lesions were monitored and photographed, as indicated in the legends, with a digital camera (Fujifilm, FinePix S1 Pro).

12.3 TMV infection:

Tobacco plants (*Nicotiana tabacum* cv *rustica*) were grown at 22°C in growth chambers programmed for a 14-hr light and 10-hr dark cycle. For high-temperature experiments, plants were transferred to 32°C Conviron chambers 2 to 3 days before inoculation. Tobacco mosaic virus (TMV) strain U1 was used at a concentration of 1

pg/mL in 50 mM phosphate buffer at pH 7.5 in all experiments. Two to three leaves were inoculated on each plant and harvested together and photographed with Nikon cool pix 5200.

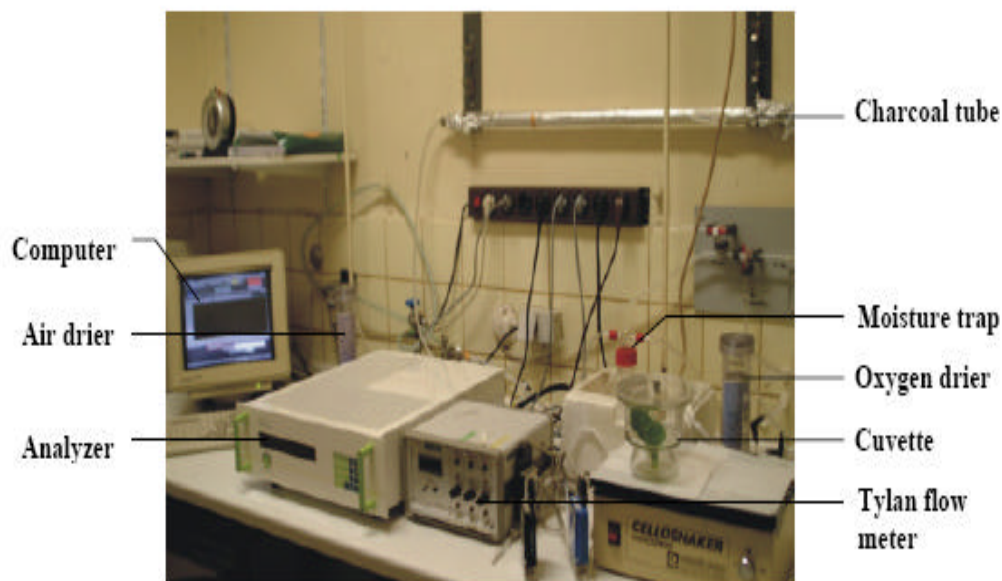


Fig 40: Chemiluminescence set up.

13 Nitrate reductase activity and nitrite content

Following different treatments, leaf or root slices were cut into 0.5 to 1 cm pieces weighed and immediately quenched in liquid nitrogen. The leaf material was ground with liquid nitrogen to a fine powder in a porcelain mortar with a pestle and 2 ml of extraction buffer (100 mM HEPES-KOH, pH 7.6, 1 mM DTT, 10 μ M FAD, 10 μ M molybdate, 15 mM MgCl_2 , 2 mM pepabloc, 50 μ M leupeptin, 50 μ M cantharidine, 0.5 % PVP, 0.5 % BSA and 0.3 % of Triton) was added to one g FW. Cantharidine (a PP2A inhibitor) was added 50 μ M in order to prevent dephosphorylation of NR. After continuous grinding until thawing, the suspension was centrifuged (14500g, 10 min, 4°C). After this centrifugation, aliquots of the extract were directly used for the

colorimetric determination of nitrite content. The remaining supernatant was desalted on sephadex G 25 spin columns (1.5 ml gel volume, 650 μ L extract, 4°C) equilibrated with the extraction buffer without the protease-inhibitors.

14. Sugar and Amino acid analysis: Major sugars (glucose, fructose and sucrose) were separated by anion-exchange chromatography (0.1 N NaOH as eluent) on a Carbowac column plus pre-column, and detected directly by pulsed amperometry (Dionex 4500 i; Dionex, Idstein, Germany). Ammonium was measured by flow injection analysis (GAT WESCAN 360; Gamma Analysentechnik, Bremerhaven, Germany). Total amino acids were measured by the ninhydrin method.

15. Northern Blotting

15.1 Total RNA isolation

At different time points following infiltration of leaves with cryptogein or with the control, the infiltrated areas were precisely cut out. The plant tissue was ground with a mortar and pestle under liquid nitrogen. The isolation of total RNA was done using the *trizol reagent* (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol.

Around 100 mg of tissue powder was mixed with 1 mL trizol. After 5 min incubation at RT, 200 μ l CHCl_3 was added. After a vigorous shaking, the lysate was centrifugated at 12000g for 15 min. Nucleic acids were precipitated with isopropanol (500 μ L). The supernatant was removed after 10 min incubation time at RT and after centrifugation (10 min at 12000g). The pellet was washed with 1 mL ethanol (75 %). After several step washing and brief centrifugation (5 min, 7500 g), the RNA pellet was dissolved in 44 μ L DEPC (diethyl pyrocarbonate)-Water and incubated for 10 min at 65°C. The

concentration of RNA was read in a spectrophotometer (Amersham Pharmacia Biotech) by measuring the absorbance at 260 nm. A ratio of $[E_{260}/E_{280}]$ with a value of 1.8-2.0 was used as a criterion for pure RNA with low protein contamination. The RNA was stored at -80°C.

15.2 Synthesis of cDNA

In the reverse transcription reaction, oligonucleotide primers are annealed to an RNA population. Reverse transcriptase extends annealed primers, creating a DNA copy (cDNA) complementary to the RNA sequences. The iScriptTM cDNA synthesis Kit (BIO-RAD, Munich, Germany) was used to cDNA production. The kit components used for the reaction were:

4 µL 5x iScript Reaction Mix

1 µl iScript Reverse Transcriptase

13 µL Nuclease-free water

2 µL RNA template (100 fg to 1 µg Total RNA)

The complete reaction mixture was incubated as follows:

5 minutes at 25°C

30 minutes at 42°C

5 minutes at 85°C

Hold at 4°C

The cDNA then served as a template for amplification by Polymerase Chain Reaction (PCR).

15.3 Polymerase Chain Reaction (PCR)

PCR was used as a tool for selective, exponential amplification of rare molecules in cDNA populations, to prove the identity of cloned fragments or to subclone the PCR products. The principle of this exponential amplification is based on the denaturation of the double stranded DNA templates where specially designed oligonucleotide molecules

(primers) anneal at the 3'- and 5'- end of the single stranded region of interest. These primers are elongated by a DNA- and Mg^{2+} - dependent DNA polymerase in the presence of free deoxynucleoside-triphosphates (dNTPs). Repetition of denaturation, annealing and amplification cycles leads to an exponentially increasing copy number of the product. Regarding primer and template parameters suitable cycling parameters were chosen. 5 min extension at 72°C after the last cycle was included to ensure that all PCR products are full length and 3' adenylated. The reactions were performed in a thermocycler (Hybaid, Heidelberg, Germany).

50 µl reaction: cDNA 2.0 µL

10x PCR magnesium buffer 5.0 µL

dNTPs (10 mM) 1.0 µL

Primer-F (10 µM) 1.0 µL

Primer-R (10 µM) 1.0 µL

Taq DNA-Polymerase (10 U/µl) 0.4 µL

H₂O 39.6 µL

Oligo Nucleotide Primers:

Name		
Nt-PR1a-F	5'-GTG CCC AAA ATT CTC AAC AAG-3'	67
Nt-PR1a-R	5'-TTA CGC CAA ACC ACC TG -3'	64

Cycling parameters:

Denaturation	Annealing	Elongation	Cycle number
95°C - 5 min			1
92°C - 1 min	55°C - 1 min	72°C - 1.5 min	35
		72°C - 5 min	1

The PCR product was subjected to agarose gel electrophoresis, which is the easiest and most common way of separating and analysing DNA. The agarose (1.4 %) was melted in 0.5x TBE (Tris-Borate EDTA) (70 mL) and mixed with 7 μ L ethidium bromide after cooling to 50°C. The PCR product (30 μ L) was mixed with an appropriate amount of loading buffer (6x) and subjected to electrophoresis for 1 h at 100 V. 100 bp DNA ladder was used as molecule size marker (peqLab, Erlangen, Germany). The DNA fragment could be checked by the fluorescence of the DNA-intercalating dye ethidium bromide under UV illumination. Afterwards, the cDNA was obtained by gel extraction according to the manufacturer's protocol (E.Z.N.A. Gel Extraction Kit; PeqLab Biotechnologie, Erlangen, Germany).

Loading Buffer (6x)

50 % (w/v) sucrose in water

100 mM EDTA

0.25 % Bromophenol blue

5x TBE (for 1 L)

54 g Tris-HCL

27.5 g Boric acid

20 mL 0.5 mM EDTA (pH 8.0)

15.4 Electrophoresis of RNA

Total RNA was used for a denaturing electrophoresis using formaldehyde-agarose gels. Formaldehyde forms unstable Schiff bases with the single imino group of guanosine residues. These adducts maintain RNA in the denatured state by preventing intra-stand Watson-Crick base pairing.

One volume of RNA-Gel loading buffer were added to 4 μ L total RNA and denatured at 65°C for 10 min. After 2 min on ice, the individual RNAs were size fractionated in a

1x MOPS agarose-formaldehyde gel (100 V), ensuring that all RNA molecules have an unfolded, linear conformation.

10 x MOPS-buffer

0.2 M MOPS (Morpholinopropane sulfuric acid)

0.01 M EDTA

0.08 M Sodium acetate pH 7.0

RNA-Gel Loading buffer

1000 μ l Formamide

100 μ l 10x MOPS

350 μ l 37 % Formaldehyde

180 μ l H₂O

100 μ l 80 % Glycerol

80 μ l 2 % Bromophenol blue solution

10 μ l 1 % Ethidium bromide

Formaldehyde-Agarose-Gel

1.5 g Agarose

90.0 ml H₂O dissolved in the microwave (1-2 min)

12.5 ml 10x MOPS

22.5 ml formaldehyde

The typical rRNA pattern could be visualized under UV-light (ImageMaster VDS, Pharmacia Biotech) due to the fluorescence of the intercalating ethidium bromide. Distinct clear bands prove the quality of the preparation. Afterwards, the gel was washed four times with H₂O (5 min) and 60 min with 10x SSC.

10x SSC

1.5 M NaCl

0.15 M sodium-Citrate

pH 7.0 (HCl)

15.5 Transfer of RNA

The transfer was achieved by "capillary blotting" after electrophoretic separation of the RNA. Large volumes of buffer (10x SSC) were drawn through the gel and the membrane (Amersham Pharmacia Biotech), thus transferring the RNA from the gel to the membrane. The RNA was fixed on the membrane by 120 mJoules of UV-light (UVStratalinker 2400, Stratagene). The membrane could then be subjected to a radioactive hybridization.

15.6 Radioactive labelling of nucleic acids and hybridization of nucleic Acids

The Oligolabelling method by "Random Primers DNA Labelling System (Invitrogen, Karlsruhe, Germany) is based on a process developed by Feinberg and Vogelstein (1984). It was used for labeling DNA restriction fragments for use as hybridization probes. The procedure was done following the manufacturers protocol. The DNA (3 μ l DNA (25 ng) + 20 μ l H₂O) was denatured (5 min, 100°C) and then mixed with dNTPs (dGTP, dATP, dTTP), 1 μ l Klenow-Polymerase (7-12 U, FPLC*pure*TM; GIBCO, Karlsruhe, Germany), 15 μ l Random Primer buffer. These "random oligomers" annealed to random sites on the DNA and then served as primers for DNA synthesis by a DNA polymerase. With 40 μ Ci

[α -³²P]dCTP present during this synthesis, labeled DNA was generated during a 1h incubation step at RT. Micro Bio-spin P-30 Tris Chromatography columns (Bio-RAD) were used to remove nucleotides which were not incorporated. The procedure was done following the manufactures protocol.

The pre-hybridization step was performed in 20 ml (65°C, 1 h) of church buffer (1 % BSA, 1 mM EDTA, 0.5 mM Na_2HPO_4 , 7 % SDS, pH 7.2). After addition of the denatured radioactive probe (see part 7.6) to a final specific activity of at least 10 $\mu\text{Ci}/\mu\text{L}$ dCTP the hybridization proceeded over night (16 h, 65°C) in a special glass tube for hybridization incubators (Biometra, Göttingen, Germany). The next day, unhybridized probe was removed by washing the membrane in several steps at 65°C with increasing stringency:

Immediately wash I : 2 x SSC, 0.1 % SDS

2x 30 min wash I

2x 15 min wash II : 0.2 x SSC, 0.1 % SDS

The membrane was sealed in a plastic wrap and exposed to a X-Ray film (Kodak X-omat DS film) at -80°C in a hyper cassette (Amersham Life Science, UK). For developing the film after an appropriate time period, the X-Ray developer (Kodak) and the X-Ray fixer (Kodak) were used.

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F Appendix

List of chemicals

Calmodulin	Sigma, Deisenhofen, Germany
c-PTI	Dojindo Laboratories, Kumamoto, Japan
c-PTIO	Alexis, Grünberg, Germany
CM	Sigma, Deisenhofen, Germany
DPI	Sigma, Deisenhofen, Germany
FMN	Sigma, Deisenhofen, Germany
L-NAME	Sigma, Deisenhofen, Germany
L-NIL	Biomol, Hamburg, Germany
L-NMMA	Calbiochem, Schwalbach, Germany
NADPH	Sigma, Deisenhofen, Germany
PTIO	Sigma, Deisenhofen, Germany
Sodium Lime	Merck, Darmstadt, Germany
SNP	Merck, Darmstadt, Germany
Tetrahydrobiopterin	Sigma, Deisenhofen, Germany
Myxothiazol	Sigma, Deisenhofen, Germany
Enzymes:	
Catalase	Sigma, Deisenhofen, Germany
Nitrate reductase from corn	Sigma, Deisenhofen, Germany
	NECi, Lake Linden, MI
Nitric oxide synthase (Mouse recombinant)	Axxora
SOD	Sigma, Deisenhofen, Germany
Gas:	
Nitric oxide 500 ppb in nitrogen	Messer, Griesheim, Darmstadt, Germany
Nitric oxide 100 ppm in nitrogen	Messer, Griesheim, Darmstadt, Germany

Abbreviations

%	percent
°C	degree Celsius
A	Adenine
ABA	Abscisic Acid
AMP	Adenosine-5'-monophosphate
AOX	Alternative Oxidase
ATP	Adenosine-5'-triphosphate
BH ₄	Tetrahydrobiopterin
BSA	Bovine Serum Albumin
C	Cytosine
cADPR	cyclic ADP Ribose
cDNA	Complementary DNA
cGMP	cyclic Guanosine 3',5'-Monophosphate
CHX	Cycloheximide
Cm	centimeter
CM	Carboxymethylamine
cNR	constitutive Nitrate Reductase
c-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl
DAF-2	4,5-diaminofluorescein
DAF-2DA	4,5-diaminofluorescein diacetate
DAF-2T	4,5-diaminofluorescein triazole
dATP	deoxyadenosine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DANN	Deoxyribonucleic Acid
dNTP	2'-desoxyribonucleosid-5'-triphosphate

DPI	Diphenyleneiodonium
DTT	1,4-dithiotreitol
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediamine-tetraacetate
e.g.	exempli gratia (= for example)
EPR	Electron Paramagnetic Resonance
eNOS	endothelial Nitric Oxide Synthase
FAD	Flavin Adenine Nucleotide
FMN	Flavin Mononucleotide
FW	Fresh Weight
G	gram
G	Guanine
GC	Guanylate Cyclase
GDC	Glycine Decarboxylase
H	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HQI	Housing Quality Indicators
HR	Hypersensitive Response
iNOS	inducible Nitric Oxide Synthase
iNR	inducible Nitrate Reductase
IP	Inactivator Protein
JA	Jasmonic Acid
Kb	kilo base
kBq	kiloBecquerel
kDa	kilo Dalton
L	Liter
Lb	Leghemoglobin

L-NAME	N ^ω -nitro-L-arginine methyl ester hydrochloride
L-NIL	L-N ⁶ -(1-Iminoethyl)-lysine, acetate
L-NMMA	NG-Monomethyl-L-arginine, Monoacetate salt
LS	Linsmaier & Skoog
M	meter
M	Molar
MAPK	Mitogen Activated Protein Kinase
Max	maximal
MES	4-morpholinic-ethanesulfonic acid
Mg	milligram
Min	minutes
mL	milliliter
mm	millimeter
mM	millimolar
MoCo	Molybdenum Cofactor
MOPS	4-morpholinic-propansulfonic acid
mRNA	messenger RNA
n	Number of samples
N ₂	Nitrogen
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
N.D.	Non Detected
Ng	nanogram
NI-NOR	Nitrite: NO-reductase
NiR	Nitrite Reductase
nNOS neuronal	Nitric Oxide Synthase

NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase
NRA	Nitrate Reductase Activity
PAL	Phenylalanine Ammonia Lyase
PAR	Photosynthetic Active Radiation
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PM	Plasma Membrane
Pmol	picomol = 10 ⁻¹² mol
PMS	Phenazine Methosulphate
PMT	Photomultiplier Tube
Ppb	part per billion
Ppm	part per million
Ppt	part per trillion
PR	Pathogenesis related
PTIO	2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
Rpm	rounds per minute
RSNO	S-nitrosothiols
RT	Room Temperature
S	second
SA	Salicylic Acid

SAR	Systemic Acquired Resistance
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase
SSC	Sodium Chloride/Sodium Citrate
T	Thymine
Taq	Thermus aquatic
TBE	Tris-borate EDTA
T _m	melting Temperature
TMV	Tobacco Mosaic Virus
U	Unit
μCi	micro-Curie
μL	micro-Liter
μM	micro-Molar
UV	Ultra-Violet
V	Volt
w/v	weight/volume
WT	Wild Type
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase

Papers published

- 1) Stoimenova M, Igamberdev AU, **Gupta KJ**, Hill RD: Nitrite driven anaerobic ATP synthesis by barley and rice root mitochondria. *Planta* 2007 July 226(2) 465-474 (Evaluated by Faculty of 1000 Biology)

- 2) Kaiser WM, **Gupta KJ**, Planchet E: Higher plant mitochondria as source of NO. Nitric Oxide in Plant Growth Development and Stress Physiology Lamattina L, Polacco JC (Eds) *Plant Cell monographs* (2007) pp 1-14

- 3) **Gupta KJ**, Stoimenova M, Kaiser WM: In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, *in vitro* and *in situ*. *Journal of Experimental Botany* 2005 Oct: 56(420):2601-9

- 4) Planchet E, **Gupta KJ**, Sonoda M, Kaiser WM. Nitric oxide emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport. *The Plant Journal* 2005 41, 732–743

Manuscripts under preparation/ submitted:

Gupta KJ and Kaiser WM: Absence of nitric oxide synthase (NOS) activity in roots and root mitochondria (in preparation)

Gupta KJ, Mishina T, Zeier J, Kaiser WM: Hypersensitive response elicited by *pseudomonas syringae* is modified by ammonium vs Nitrate nutrition (in preparation)

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Gupta KJ, Mishina T, Zeier J, Kaiser WM: Hypersensitive response elicited by *Pseudomonas syringae* is modified by ammonium vs Nitrate nutrition (Plant NO club meeting Verona Italy 2006).

Gupta KJ and Kaiser WM: Mitochondrial pathways of nitric oxide production in plants: Some new Insights (Plant NO club meeting Verona Italy 2006)

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation in allen Teilen selbst angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

Ich erkläre weiterhin, dass ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg,

October 2007