

**NITRIC OXIDE PRODUCTION  
BY TOBACCO PLANTS AND CELL CULTURES  
UNDER NORMAL CONDITIONS AND UNDER STRESS**

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*À mes parents,  
À Alicia et Flavie*

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## SUMMARY

Nitric oxide (NO) is a gaseous free radical involved in the regulation of diverse biochemical and physiological processes in animals. During the last decade, evidence has accumulated that NO might also play an important role as a second messenger in plants. Of special interest were observations that NO was involved in a signal chain leading to the hypersensitive response (HR) in incompatible plant-pathogen interactions. In contrast to animals, plants have probably several enzymes that may produce NO. Potential candidates are: Cytosolic nitrate reductase (NR; EC 1.6.6.1), plasma-membrane (PM)-nitrite: NO reductase (Ni:NOR), nitric oxide synthase (NOS; EC 1.14.13.39) and Xanthine dehydrogenase (XDH; EC 1.1.1.204). The major goal of this work was to quantify NO production by plants, and to identify the enzymes responsible for NO production. As a major method, NO production by tobacco leaves or cell suspensions was followed under normal, non-stress conditions, and under biotic stress, through on-line measurement of NO emission into the gas phase (chemiluminescence). 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. Induction of HR in tobacco leaves and in cell suspensions was achieved using the fungal peptide elicitor cryptogein.

Non-elicited leaves from nitrate-grown plants showed a typical NO-emission pattern where NO-emission was low in dark, higher in the light and very high under dark-anaerobic conditions. Even at maximum rates, NO production *in vivo* was only a few percent of total NR activity (NRA). Consistent with that, with a solution of purified NR as a simple, “low quenching” system, NO-emission was also about 1 % of NRA. Thus, NO scavenging by leaves and stirred cell suspensions appeared small and NO-emission into purified air should give a reliable estimate of NO production.

NO-emission was always high in a NiR-deficient transformant which accumulated nitrite, and NO-emission was completely absent in plants or cell suspensions which did not contain NR. Thus, in healthy plants or cell suspensions, NO-emission was exclusively due to the reduction of nitrite to NO, mainly by cytosolic NR. In addition to nitrite, cytosolic NADH appears as an important factor limiting NO production. Unexpectedly, plants (in

absence of NR) were able to reduce nitrite to NO under anaerobic conditions through an unknown enzyme system that was not a MoCo-enzyme and was cyanide-sensitive.

When infiltrated into leaves at nanomolar concentrations, the fungal elicitor cryptogein provoked cell death in tobacco leaves and cell suspensions. The HR could be prevented by the NO-scavengers PTIO or c-PTIO, suggesting that NO production was indeed required for the HR. However, the product of the reaction of c-PTIO with NO, c-PTI, also prevented cell death without quenching NO emission. Thus, prevention of cell death by c-PTIO is no proof for an involvement of NO.

No differences were found in the HR induction between NR-free plants and/or cell suspensions and WT plants. Thus, NR appears not necessary for the HR. Further, and in contrast to literature suggestions, a continuously high NO-overproduction by a NiR-free mutant did not interfere with the development of the HR. Most surprisingly, no additional NO-emission from tobacco leaves was induced by cryptogein at any phase of the HR. In contrast, some NO-emission, paralleled by nitrite accumulation, was detected 3-6 h after cryptogein addition with nitrate grown cell suspensions, but not with NR free, ammonium-grown cells. Thus, induction of NO-emission by cryptogein appeared somehow correlated with NR and nitrite, at least in cell suspensions. But since cryptogein induced the HR even in NR-free cell suspensions, this nitrite-related NO- emission was not required for cell death. NOS inhibitors neither prevented cell death nor did they affect nitrite-dependent NO-emission. Thus, in total these data question the often proposed role of NO as a signal in the HR, and of NOS as source for NO.

## ZUSAMMENFASSUNG

Stickstoffmonoxid (NO) ist ein gasförmiges freies Radikal. In tierischen Geweben ist NO an der Regulation vieler physiologischer Prozesse beteiligt. In den letzten zehn Jahren wurde immer wahrscheinlicher, dass NO auch in Pflanzen als „second messenger“ fungiert. Besonderes Interesse fanden Berichte, dass NO als intermediäres Signal bei der Induktion der hypersensitiven Antwort (HR) von Pflanzen auf Pathogene involviert ist. Im Gegensatz zu Tieren haben Pflanzen wahrscheinlich eine Reihe verschiedener Systeme, die NO produzieren können. Potentielle Kandidaten dafür sind: cytosolische Nitratreduktase (NR; EC 1.6.6.1), PM-gebundene Nitrit: NO Reduktase (Ni:NOR), NO-Synthase (NOS; EC 1.14.13.39) und Xanthindehydrogenase (XDH; EC 1.1.1.204). Das Ziel dieser Arbeit bestand darin, die NO-Produktion von Pflanzen zu quantifizieren und die beteiligten enzymatischen Schritte zu identifizieren. Als wichtigste Methode zur NO-Messung wurde die Chemilumineszenz verwendet, mit der die NO Emission aus Pflanzen, Zellsuspensionen oder Enzymlösungen in NO-freie Luft oder N<sub>2</sub> in Echtzeit verfolgt werden konnte. Wir benutzten für unsere Analyse: Tabak Wildtyp (*N. tabacum* cv *Xanthi* oder cv *Gatersleben*) und Zellsuspensionskulturen davon, NR-freie Mutanten oder WT Pflanzen, die auf Ammonium angezogen wurden um NR-Induktion zu vermeiden, Pflanzen die auf Wolframat an Stelle von Molybdat wuchsen um die Synthese funktionierender MoCo-Enzyme zu unterdrücken, und eine NO-überproduzierende, Nitritreduktase (NiR)-defiziente Transformante.

Normale Blätter von nitraternährten Pflanzen zeigten ein typisches NO-Emissionsmuster, bei dem die NO-Emission im Dunkeln niedrig, im Licht viel höher, und unter anoxischen Bedingungen im Dunkeln mit weitem Abstand am höchsten war. Aber selbst nach Erreichen maximaler Raten war die NO-Emission höchstens 1 % der extrahierbaren NR Aktivität. Auch eine Lösung hochgereinigter Nitratreduktase produzierte NO aus den Substraten Nitrit und NADH, und auch hier war die Rate der NO-Emission nur maximal 1 % der vorhandenen NR-Aktivität. Dieses übereinstimmende Verhältnis von NR Aktivität und NO-Emission in Blättern, Zellsuspensionen und einer NR-Lösung zeigt an dass die NO-Löschung nur gering war und dass deshalb die NO-Emissionsmessung eine zuverlässige Methode zur Quantifizierung der NO Produktion sein sollte.

Die NO-Emission aus einer NiR-defizienten, nitritakkumulierenden Transformante war immer sehr hoch. NR-freie Pflanzen oder Zellsuspensionen produzierten dagegen

normalerweise kein NO, woraus geschlossen werden konnte, dass hier NR die einzige NO-Quelle war. Die Rate war in der Regel korreliert mit der Nitritkonzentration, aber cytosolisches NADH erschien als ein weiterer wichtiger limitierender Faktor. Überraschenderweise reduzierten aber auch NR-freie Pflanzen oder Zellkulturen unter anoxischen Bedingungen Nitrit zu NO. Das beteiligte Enzymsystem war kein MoCo-Enzym und war Cyanid-sensitiv.

Der pilzliche Elicitor Cryptogein induzierte nach Infiltration in Blätter oder nach Zugabe zu Zellsuspensionen bereits in nanomolaren Konzentrationen den Zelltod. Diese Antwort wurde verhindert oder zumindest stark verzögert durch den NO-Scavenger PTIO oder c-PTIO. Die Schlussfolgerung war zunächst, das NO tatsächlich an der HR-Induktion involviert war. Da aber das Reaktionsprodukt von c-PTIO und NO, c-PTI, den HR ebenfalls verhinderte ohne jedoch NO zu löschen, scheint die weit verbreitete Verwendung von c-PTIO und seinen Derivaten für die Beweisführung einer Beteiligung von NO zumindest fragwürdig.

Der HR wurde unterschiedslos sowohl in WT-Pflanzen als auch in NR-freien Pflanzen bzw. Zellsuspensionen induziert. NR ist also offensichtlich für den HR nicht erforderlich. Im Gegensatz zur publizierten Literaturdaten verhinderte auch eine kontinuierliche hohe Überproduktion von NO die Ausprägung des HR nicht. Besonders überraschend war der Befund, dass trotz der Hemmung des HR durch PTIO keinerlei Cryptogein-induzierte NO Produktion in Blättern messbar war. Allerdings wurde in nitraternährten Zellsuspensionskulturen ca. 3-6 h nach Cryptogein-Gabe eine -wenn auch geringe- NO-Emission beobachtet, die von einer Nitritakkumulation begleitet war. Beides blieb in Ammonium-ernährten Kulturen aus. Hier schien also eine gewisse Relation zwischen Cryptogein-induzierter NO Emission, NR und Nitrit zu bestehen, die im Detail noch nicht verstanden ist. Da der Zelltod aber auch in NR-freien Zellsuspensionskulturen auftrat, besteht offensichtlich kein kausaler Zusammenhang zwischen dieser NO-Emission, Nitritakkumulation und der Cryptogein-Wirkung. Da NOS-Inhibitoren weder den Zelltod noch die nitritanhängige NO-Emission verhinderten, scheint eine NOS-artige Aktivität ebenfalls keine Rolle zu spielen. Insgesamt werden damit die in der Literatur etablierte Rolle von NO als Signal beim HR und die Rolle von NOS als NO-Quelle stark in Frage gestellt.

## A INTRODUCTION

Nitrogen monoxide or nitric oxide (NO) was discovered by Joseph Priestley in 1772. For more than two centuries, this colourless and odourless gas has been considered highly toxic. Over the past 20 years, the free radical NO has ignited enormous interest in the scientific community. First, in 1980, Furchgott and Zawadzki reported that endothelial cells released a substance which is responsible for the relaxation of vascular smooth muscle (they called this substance “endothelium-derived relaxing factor”: EDRF). Then in 1987, Palmer *et al.* suggested that EDRF is NO, which is produced by the oxidation of L-arginine. In 1992, *Science* named NO “Molecule of the Year” because of its widespread biological significance (Koshland, 1992). Robert F. Furchgott, Louis J. Ignarro and Ferid Murad received in 1998 the Nobel Prize in Physiology and Medicine for their discoveries concerning "nitric oxide as a signalling molecule in the cardiovascular system". The biology of NO and its clinical importance are rapidly evolving as more and more research papers are reported from different centers. NO has been identified as a critical signalling molecule in maintaining blood pressure in the cardiovascular system, stimulating host defenses in the immune system, regulating neuronal transmission in the brain, regulating gene expression, platelet aggregation, relaxation of smooth muscle, learning and memory, male sexual function, cytotoxicity and cytoprotection, among others (Moncada *et al.*, 1991; Lipton *et al.*, 1993).

### 1 Physiological chemistry of NO

The chemistry of NO is the most important determinant of its function in biological systems and may be separated into two basic categories, i) direct effects and ii) indirect effects, chiefly based on the concentration of NO (Wink and Mitchell, 1998). At sites where high and sustained amounts of NO are produced, the indirect effects prevail. They arise rapidly and from non-enzymatic interactions of NO with oxygen and superoxide ( $O_2^-$ ) giving reactive nitrogen species and reactive oxygen species (ROS), prior to participating in chemical modification of biological targets. Conversely, direct effects are reactions in which NO reacts directly with metal complexes (*e.g.* heme, guanylate cyclase (GC)), non-

heme iron proteins, zinc and copper proteins and powerful free radicals. These reactions are generally fast and require low concentrations of NO ( $< 1 \mu\text{M}$ ) (Miranda *et al.*, 2000).

### ***1.1 Chemistry and reactions of NO***

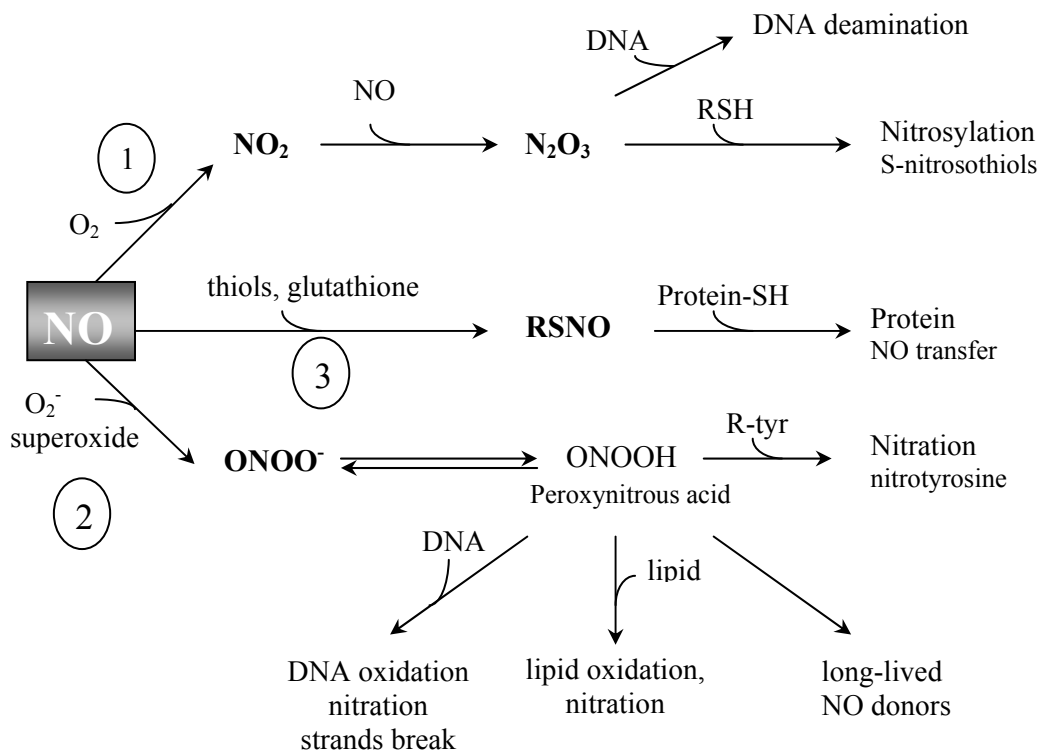
The universal and unique role of the free radical NO as a biological signalling molecule is based on its physico-chemical properties which determine the mechanisms of the interaction with its targets and the nature of its movement. Its hydrophobicity enables not only a rapid diffusion in a physiological environment but also the unhindered passage through lipid membranes, such as cell plasma membrane. NO is a free radical lipophilic diatomic gas under atmospheric conditions. It is often said that NO has a relatively short half-life in biological systems, in the order of 5-15 seconds (Lancaster, 1997); however, *in vivo*, the half-life of NO may be much longer. Because of its free radical nature, it can adopt an energetically more favourable electron structure by gaining or losing an electron, so that NO can exist as three interchangeable species: the free radical nitric oxide ( $\text{NO}^\bullet$ ), the nitrosonium cation ( $\text{NO}^+$ ), and the nitroxyl anion ( $\text{NO}^-$ ) (Stamler *et al.*, 1992; Wojtaszek, 2000).

Compared to other free radicals, NO has a rather low overall reactivity. It reacts predominantly with molecules that have orbitals with unpaired electrons, which are typically other free radicals or transition metals like heme iron. Neutral NO has a single electron in its  $2P-\pi$  antibonding orbital (Stamler *et al.*, 1992). Although it was mistakenly believed that the oxidation of NO by molecular oxygen is the main pathway of NO degradation, this reaction is of minor importance under physiological conditions (Figure 1). The reaction of NO with  $\text{O}_2$  is termed NO autoxidation. In the gas phase and in hydrophobic layers of cellular membranes, NO autoxidation initially produces  $\text{NO}_2$ , which then reacts with an additional NO molecule to form  $\text{N}_2\text{O}_3$  (dinitrogen trioxide) which can efficiently nitrosate cellular thiols and indirectly deaminate DNA.

The reaction between NO and  $\text{O}_2^-$ , in gas phase and in water solution, generates  $\text{ONOO}^-$  (peroxynitrite) (Figure 1). The half-life of  $\text{ONOO}^-$  is of the order of one second under physiological conditions. The formation of the two radicals, NO and  $\text{O}_2^-$ , depends on a delicate balance between the production of  $\text{O}_2^-$  and superoxide dismutase (SOD) and NO synthesis and consumption (Koppenol, 1998). At physiological pH,  $\text{ONOO}^-$  equilibrates

rapidly with peroxynitrous acid (ONOOH), which spontaneously decomposes to  $\text{NO}_3^-$  or to the highly reactive hydroxyl radical  $\text{HO}^\bullet$ . Although neither NO nor  $\text{O}_2^-$  is a strong oxidant, ONOO<sup>-</sup> is a potent and versatile oxidant capable of initiating lipid peroxidation and oxidizing thiols or lipid soluble antioxidants (Darley-Usmar *et al.*, 1995). NO reacts rapidly with these LOO and/or lipid alkoxy radicals, resulting in LOONO or LONO formation, respectively, which in turn leads to chain termination. However, no direct evidence has been given that ONOO<sup>-</sup> is formed *in vivo*. ONOO<sup>-</sup> is capable of nitrating tyrosine residues of proteins. The presence of nitrotyrosine has been measured routinely as a marker for ONOO<sup>-</sup> formation *in vivo*.

NO can also react with thiols (S-nitrosation) to produce S-nitrosothiols (RSNO) (Figure 1). NO may be stored and shuttled by transnitrosation of proteins with thiols groups. The reactions of NO with glutathione produce S-nitrosoglutathione for the storage or transfer.



**Figure 1:** Reactions of the free radical NO and reactive nitrogen oxide species.

1- Reactions of NO with  $\text{O}_2$  producing  $\text{N}_2\text{O}_3$  lead indirectly to DNA deamination.  $\text{N}_2\text{O}_3$  also reacts with thiols producing S-nitrosothiols (RSNOs). 2- NO reacts with  $\text{O}_2^-$  to form ONOO<sup>-</sup>. 3- The bioactive formation of S-nitrosothiols for the storage, transfer and production of NO (from Durzan, 2002).

In addition, NO is extremely susceptible to both oxidation and reduction. One electron oxidation of NO leads to NO<sup>+</sup>. NO<sup>+</sup> mediates electrophilic attack on reactive sulphur, oxygen, nitrogen and aromatic carbon centers. This chemical process is referred to as “nitrosation”. Oxidative stress can also potentially be mediated by NO<sup>-</sup>, generated by one electron reduction of NO. The physiological significance of NO<sup>-</sup> has not been clarified. NO<sup>-</sup> is also believed to react with FE (III) heme and to mediate sulfhydryl oxidation of target proteins. The formation of NO<sup>-</sup> and molecular oxygen (O<sub>2</sub>) *in vivo* could also be important for cellular ONOO<sup>-</sup> production (Henry and Guissani, 1999).

## ***1.2 Molecular targets of NO in plants***

The multiple redox forms of NO species and oxidation states of iron suggest an enormous number of possible interactions. Indeed, studies on the interaction of NO with metalloproteins *in vitro* indicated that NO has the capability of complexing enzyme-bound ferrous ions, including heme-associated iron. Hemoglobin has long been considered as a prototype of hemo-proteins. The interplay between hemoglobins and NO has been shown by studies in mammals and microbes (Gow and Stamler, 1998, Hausladen *et al.*, 1998), and this reaction is considered as a key mechanism in detoxification of NO. Plants also contain hemoglobin genes (Hunt *et al.*, 2001), so it is possible that plant hemoglobins can form reversible NO complexes. These plant hemoglobins can be classified into three groups that have been categorized as symbiotic, non symbiotic and truncated hemoglobins (Dordas *et al.*, 2003b). The symbiotic-type hemoglobins, or leghemoglobins (Lb), are found in infected cells of nitrogen-fixing nodules of both legume and non-legumes. The formation of Lb-NO results in inactivation of the Lb which consequently can not fulfil its role as an oxygen carrier. Some reports have shown that NO acts as a negative regulator of nitrogen fixation due to its interaction with leghemoglobin (Herouart *et al.*, 2002). Other data indicate that modulation of NO levels results in alteration of nodule numbers (Herouart *et al.*, 2002). The second group of plant hemoglobins, also termed phytohemoglobins, are present in non-symbiotic organs of legumes and in non-legumes. The variation in NO levels in alfalfa root lines differing in their hemoglobin protein expression strongly suggested that it may be affecting turnover of NO by reaction with oxyhemoglobin to form nitrate (Dordas *et al.*, 2003a).



NO-induced alteration of mitochondrial function may be harmful for the plant cell. The mitochondrial aconitase is a constituent of the Krebs cycle, so its inactivation by NO decreases cellular energy metabolism. This aconitase, an iron-sulphur (4Fe-4S)-containing enzyme, catalyzes the reversible isomerization of citrate to isocitrate and regulates iron homeostasis, suggesting a role for NO in modulating iron levels in plants (Navarre *et al.*, 2000). The mitochondrial respiratory electron transport chain includes abundant proteins containing transition metals such as heme. In fact, NO reduces total cell respiration and inhibits the ATP synthesis (oxidative phosphorylation) by involving the inhibition of the cytochrome pathway (Yamasaki *et al.*, 2001; Takahashi and Yamasaki, 2002), thereby favouring electron flow through the alternative oxidase pathway (Millar and Day, 1996; Zottini *et al.*, 2002). This inhibition of the cytochrome pathway by NO is a potential cause of oxidative damage in mitochondria. On the other hand, NO also interacts with various key enzymes that participate in early defense, *e.g.* catalase and ascorbate peroxidase (Clark *et al.*, 2000; Klessig *et al.*, 2000) and subsequently increase the intracellular H<sub>2</sub>O<sub>2</sub> concentration in plant.

On the other hand, the heme group of the soluble form of GC is clearly one of the most sensitive and important sites of the action of NO, based on the well-established role in many NO-mediated responses. It is well-known that NO can also interact with reactive amino acids such as cysteine and tyrosine in proteins and with thiol groups present in other molecules such as glutathione (Wendehenne *et al.*, 2001). It is important to note that the chloroplast stroma contains many thiols that are functionally important for photosynthesis.

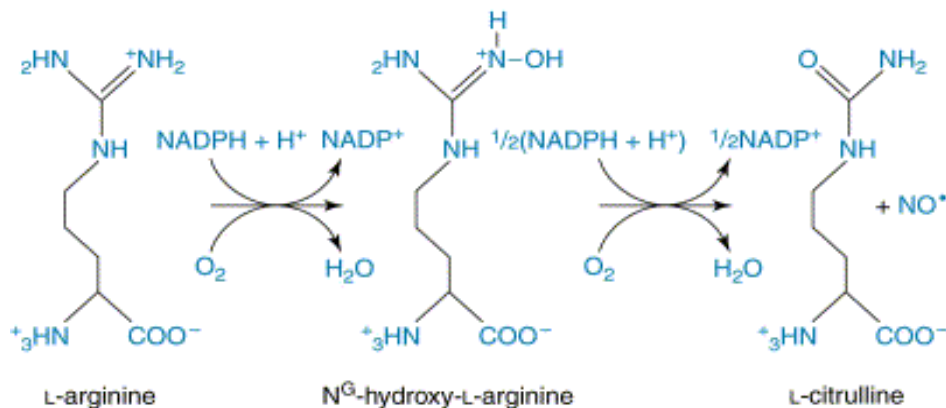
## **2 NO biosynthesis**

There are several potential sources of NO in plants and it would seem likely that production of NO will depend on the species, the cells/tissues, the conditions under which the plants are grown and, of course, the signalling pathways active under those specific conditions. There is evidence for NO production in plants from nitric oxide synthase-like enzymes, from nitrate reductase, and from other enzymatic or non-enzymatic sources.

## 2.1 Arginine-dependent nitric oxide synthase (NOS)

### 2.1.1 NOS in animals

Production and functions of NO have been intensively studied in animal physiology, often under clinical aspects. In mammalian cells, NO and L-citrulline are formed by a five-electron oxidation of one of the guanidino nitrogens of L-arginine in the presence of molecular oxygen (Groves and Wang, 2000). The reaction is catalyzed by a group of enzymes called NOS (EC 1.14.13.39) (Figure 2).

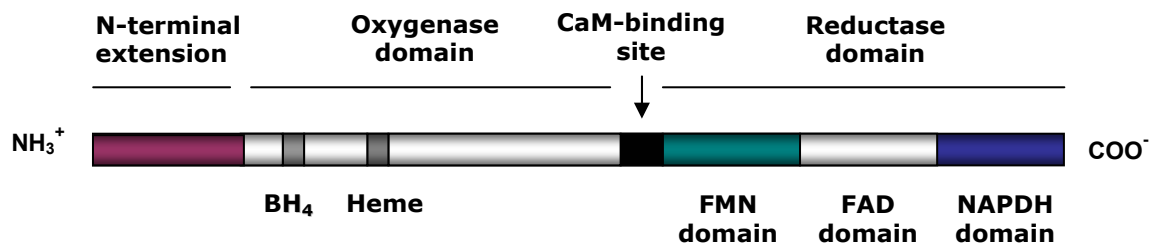


**Figure 2:** Biosynthesis of NO *via* NOS.

L-arginine is converted to NO in two successive steps: first a two-electron oxidation of L-arginine to N-hydroxy-L-arginine, which is then converted to NO and citrulline, utilizing one and half NADPH and O<sub>2</sub>. Both steps require Ca<sup>2+</sup> and calmodulin as activators and are accelerated by tetrahydrobiopterin (from Wendehenne *et al.*, 2001).

Three isoforms of NOS have been purified, cloned, and characterized from mammalian organisms: the neuronal NOS (nNOS); the inducible NOS (iNOS) and the endothelial NOS (eNOS) (Griffith and Stuehr, 1995). All NOS isoforms require for catalytic site molecular oxygen and NADPH plus cofactors (flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH<sub>4</sub>), iron protoporphyrin IX, flavin mononucleotide (FMN)) as well as the allosteric activator calmodulin. Structures of the three NOS isoforms are very similar and appear to exist *in vivo* as homodimers with a topology of each subunit having two distinct domains closely related to cytochrome P450s. The oxygenase domain contains the heme and tetrahydrobiopterin binding sites. The reductase domain contains binding sites

for FAD, FMN and NADPH, while a calmodulin binding sites lies between the oxidase and reductase domains (Figure 3). The NOS enzyme is a homodimer of approximately 260-kD. However, the formation of NO by nNOS and eNOS is  $\text{Ca}^{2+}$  dependent while iNOS activity is  $\text{Ca}^{2+}$  independent (Nathan and Xie, 1994). Evidence has been presented for production of  $\text{O}_2^-$  anion radical from both the oxygenase domain and the flavoprotein domain of neuronal NOS, leading to the generation of  $\text{ONOO}^-$  or other reactive species (Pou *et al.*, 1992; Stuehr *et al.*, 2001).



**Figure 3:** Model of the domain organization and the principle catalytic pathways of NOS.

A subunit of NOS holds a heme containing oxygenase domain (binds L-arginine in the presence of  $\text{BH}_4$ ) and a reductase domain (binds  $\text{Ca}^{2+}$ /CAM, FMN, FAD, NADPH).

### 2.1.2 NOS homologs in plants

Up to now, neither the genes nor proteins responsible for NOS activity have yet been isolated from plants, in spite of the fact that sequencing of *arabidopsis* genome is completed (*Arabidopsis* Genome Initiative, 2000). Nevertheless, it is widely assumed that NO production is catalyzed by NOS. Very recently, Chandok *et al.* (2003) demonstrated that the pathogen-inducible NOS in plants is a variant of the P protein of the glycine decarboxylase complex (GDC). The authors showed that inhibitors of the P protein of GDC block iNOS activity, and that an *Arabidopsis* variant P protein expressed in *E.coli* has NOS activity. The plant enzyme shares many biochemical properties with animal NOSs. Despite this, only a few of the critical motifs used by animal NOSs for NO production can be recognized in the variant P sequence. Moreover, like the animal NOS, it requires  $\text{BH}_4$ , FAD, NADPH,  $\text{O}_2$  as well as  $\text{Ca}^{2+}$  and CaM. However, so far, tetrahydrobiopterin has not been identified in plants. As already described previously, the production of NO in animal cells is catalyzed by different NOS isoforms, either constitutive or inducible, and plants may possess different NOS isoforms as well. Thus,

iNOS may not be the only source of NO in plants and the existence of constitutive NOS would help to explain the basal NOS activity detected under diverse conditions as in *Lupinus albus* nodules (Cueto *et al.*, 1996), in the legume *Mucuna hasjao* (Ninnemann and Maier, 1996), in pea leaf peroxisomes (Barroso *et al.*, 1999) and in soluble fractions of root tips and young leaves of maize seedlings (Ribiero *et al.*, 1999). The localization of this plant iNOS and mechanisms for the regulation of its activity during the disease resistance are still unknown. However, the presence of NOS in the cytosol, in the nucleus of maize cells (Ribiero *et al.*, 1999), in peroxisomes of pea plants (Barroso *et al.*, 1999) and in chloroplasts (Barroso *et al.*, 1999) has been reported.

Another approach to verify the presence of NOS in plants is to use commercially available NOS inhibitors. NO generation by plant cells was shown to be sensitive to inhibitors like L-NMMA or guanidine (Cueto *et al.*, 1996; Ninnemann and Maier, 1996; Delledonne *et al.*, 1998; Barroso *et al.*, 1999; Foissner *et al.*, 2000; Garces *et al.*, 2001; Chandok *et al.*, 2003).

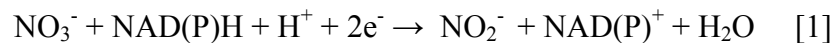
Using mouse nNOS antibody, Kuo *et al.*, (1995) revealed two distinct immunoreactive protein bands of 89.7 and 57.5 kDa in wheat-germ extracts. A single band of 105.4 kDa was also detected in pea embryonic axes using rabbit nNOS antibody (Sen and Cheema, 1995). Furthermore, Ribiero *et al.* (1999) found a 166-kDa protein using mouse iNOS antibody and several immunoreactive proteins ranging from 51 to 166 kDa using rabbit nNOS antibody in maize embryonic axes and young leaves, respectively. Despite the now fairly convincing immunological data for the presence of NOS in plants, caution should be exercised, as false positives can occur (Lo *et al.*, 2000; Butt *et al.*, 2003). These authors have clearly shown that the majority of immunoreactive plant proteins are not directly related to NO metabolism in plants.

Bredt *et al.* (1991) demonstrated that mammalian NOSs share very high sequence homology with cytochrome P450 reductases and lack regions of homology specific to NO which can make it difficult to discriminate between NOS and cytochrome P450 reductase sequences. However, it is important to note that a complementary DNA sequence with high homology to PIN, a protein inhibitor of NOS, has been identified in plants (Jaffrey and Snider, 1996).

## 2.2 Nitrate reductase (NR)

### 2.2.1 Nitrate assimilation in higher plants

Higher plants acquire the majority of their nitrogen from the environment by nitrate assimilation. These plants assimilate nitrate into organic nitrogen compounds by a highly regulated process (Crawford, 1995). The enzyme which catalyses the first step in the nitrate-reducing pathway, is nitrate reductase (NR; 1.6.6.1) and is localised in the cytosol (Oaks, 1994). However, there is growing evidence that NR can also be located at the outside of the plasma membrane (Stöhr *et al.*, 2001). The reduction catalysis [1] leads to the production of nitrite and needs NADH/NADPH or both (bispecific) as electron donors.



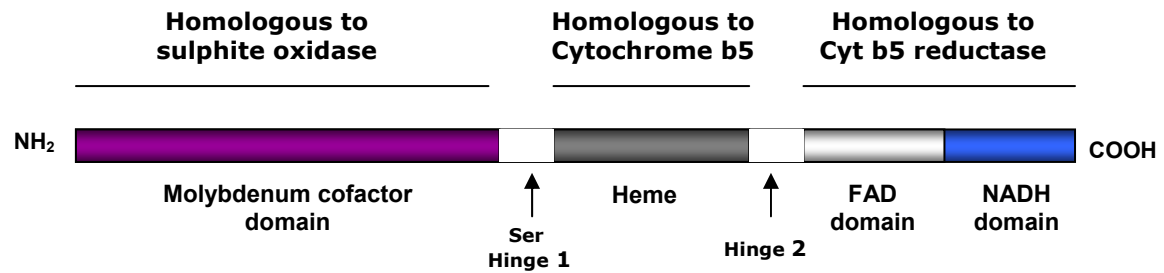
It is generally assumed that this reaction represents the rate limiting step in the N assimilation pathway (Campbell, 1999), therefore NR is considered to be the key enzyme for assimilatory nitrogen metabolism (Huber *et al.*, 1996; Kaiser *et al.*, 1999; Stitt, 1999). The first product of nitrate reduction is nitrite, which is reduced to ammonium in plastids. This process [2] is catalysed by nitrite reductase (NiR; EC 1.7.7.1).



Subsequently, ammonium is incorporated into amino acids, catalysed primarily by glutamine synthetase and glutamate synthase (for reviews, see Crawford, 1995; Lam *et al.*, 1996; Stitt, 1999).

#### 2.2.1.1 Structural and functional characteristics of NR

NR from higher plants appears to be a dimer of identical subunits of the size 100-115 kDa (Solomonson and Barber, 1990). The localization of each enzyme domain is defined by sequence homology with other enzymes (Figure 4): cytochrome b<sub>5</sub> reductase for the FAD domain, cytochrome b<sub>5</sub> for the heme domain and sulphite oxidase for the molybdenum cofactor (MoCo) domain (Crawford *et al.*, 1988; Neame and Barber, 1989).



**Figure 4:** Topology of higher plant NR.

The NR regulatory phosphorylation site is shown in Hinge 1.

Two forms of NR have been found in plants and the most common form is a NADH-specific NR (EC 1.6.6.1). A NAD(P)H-bi-specific NR (1.6.6.2) has also been described in several species, either as a second isoform along with NADH-specific NR as in maize, barley, rice and soybean (Kleinhofs and Warner, 1990), or as the sole isoform, as in *Betula pendula* (Friemann *et al.*, 1991). A NAD(P)H-specific form (EC 1.6.6.3) has not been found in higher plants but is present in fungi and mosses (Padidam *et al.*, 1991).

#### 2.2.1.2 Regulation of NR

##### ■ Transcriptional regulation of NR

NR gene expression depends on the presence of exogenously applied nitrate. For instance, reporter gene expression using an NR promoter was shown to be inducible by nitrate (Campbell, 1996; Daniel-Vedèle and Caboche, 1996), demonstrating transcriptional regulation. This induction is fast (within minutes) and requires very low concentrations of nitrate (< 10 $\mu$ M), suggesting that nitrate is actually sensed more as a signal molecule than a nutrient (Crawford, 1995). In the absence of nitrate, NR mRNA was not detected in leaves and only low levels were present in barley roots, but could be detected in roots and leaves within 40 min after supplying nitrate to roots (Melzer *et al.*, 1989).

In addition to nitrate, light is an important signal for NR regulation, at both transcriptional and post-transcriptional levels. Complex patterns of transcript and protein levels occur diurnally. The level of NR mRNA decreased when mature plants were put in darkness (Bowsher *et al.*, 1991) and increased when for instance, etiolated plants were transferred

into light (Mohr *et al.*, 1992); this induction was mediated by phytochrome (Pilgrim *et al.*, 1993). During a 24 h light-dark regime, it has been reported that in tomato and tobacco, NR-mRNA usually increases towards the end of the night and decreases during the day (Galangau *et al.*, 1988). In maize, leaf NR mRNA-levels are low during the night, peak within 2 h after transfer to the light, and decrease thereafter (Li and Oaks, 1993). NR activity (NRA) decreased rapidly to about 15 % of the control with a half-time of only 2 min after darkening of spinach leaves (Riens and Heldt, 1992). This induction by light was mediated by the stimulation of carbohydrate synthesis through photosynthesis. Kaiser and Förster (1989) demonstrated that NR activity in spinach leaves was rapidly decreased within minutes when rates of photosynthesis were decreased, *e.g.* by closure of stomata or after lowering the external CO<sub>2</sub> concentration (Kaiser and Brendle-Behnisch, 1991). Addition of sucrose, glucose or fructose (0.2 M) to the incubation media induced NR protein activity and mRNA in dark (Vincentz *et al.*, 1993). The NR induction is inhibited not only by glutamine (Deng *et al.*, 1990) but also by reduced N-compounds such as ammonium (Aslam *et al.*, 1997) or amino acids as asparagines, which are the most potent inhibitors (Sivasankar and Oaks, 1996).

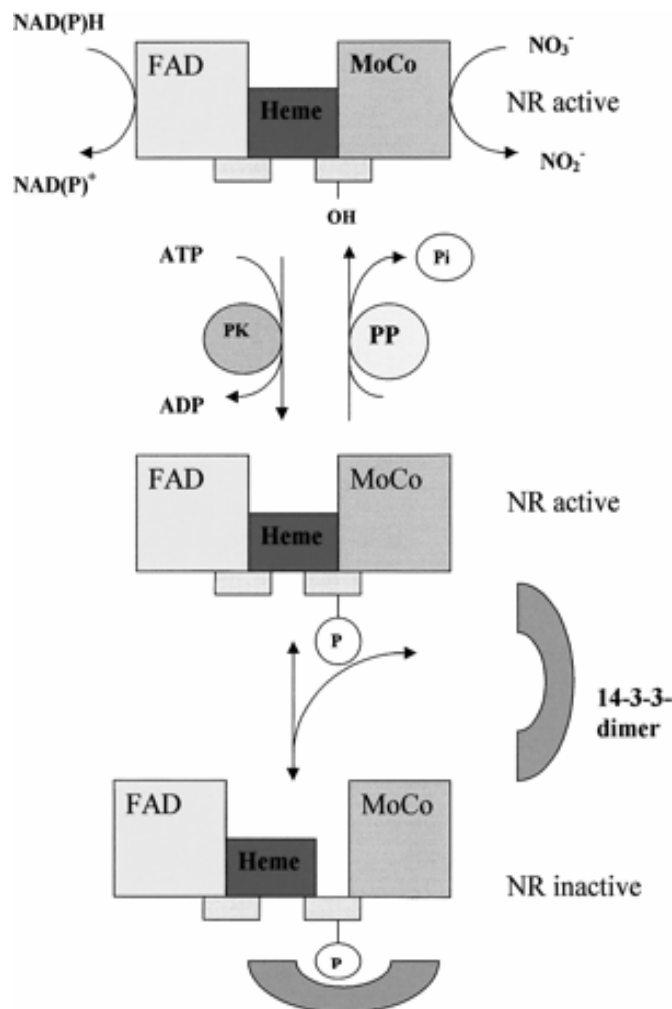
#### ■ Post-transcriptional regulation of NR

Post-transcriptional regulation of NR is important for the short-term coupling between photosynthesis and nitrate reduction. Following transfer of wild-type (WT) tobacco plants in light, NR mRNA typically shows a rapid decrease, but NR proteins and NRA rise during the first hours to reach a peak in the middle of the photoperiod (Scheible *et al.*, 1997). Later in the photoperiod, there is a decline of NR protein and activity in WT plants (Geiger *et al.*, 1998). When transgenic plants that express the tobacco *Nia2* cDNA under the control of the constitutive 35S promoter are transferred to darkness, there is a decrease of NR protein and activity, even though NR mRNA remains high (Vincentz and Caboche, 1991).

A major breakthrough in the past decade has been the demonstration of light-mediated post-translational control of NR expression (Kaiser and Förster, 1989; Kaiser and Brendle-Behnisch, 1991; Huber *et al.*, 1992; Kaiser *et al.*, 1992; MacKintosh, 1992). This regulation has been shown to operate by reversible protein phosphorylation: by assaying spinach NRA in the presence of Mg<sup>2+</sup>, a reversible inactivation of NR has been demonstrated when plants are submitted to water stress, low CO<sub>2</sub> levels or transferred to

the dark (reviewed by Kaiser *et al.*, 1999; Kaiser *et al.*, 2002b). Inactivation of NR is linked with phosphorylation both *in vitro* and in *planta* and can be seen when NRA is measured in the presence of  $Mg^{2+}$  in the millimolar range; indeed EDTA reactivates the enzyme, which allows an easy measurement of NR activation state (ratio between NRA measured with  $Mg^{2+}$  and NRA measured in the presence of EDTA).

The proteins involved in the activation mechanism of spinach NR were identified by different groups, and a regulation model (Figure 5) was proposed (Kaiser and Huber, 2001).



**Figure 5:** Model of the post-translational modulation of NR.

The enzyme consists of three different functional and structural domains (labelled in different shades of grey), which are connected by two hinge regions (in dark 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 (from Kaiser and Huber, 2001)

After transfer from light to dark, NR in leaves is first phosphorylated on at least one serine residue (serine 543) in the first hinge separating the MoCo and the haem domains. This serine residue is conserved among higher plants NRs (Douglas *et al.*, 1995; Bachmann *et*



*al.*, 1996b). Phosphorylated NR is still active and becomes inactivated upon the binding of a NR inactivator protein (IP) (Spill and Kaiser, 1994; Glaab and Kaiser, 1995; Bachmann *et al.*, 1995; MacKintosh *et al.*, 1995). IP was identified as a mixture of proteins belonging to the 14-3-3 family, and it was suggested that these 14-3-3-proteins interact with the regulatory phosphorylation site of NR (Bachmann *et al.*, 1996a; Moorhead *et al.*, 1996; Athwal *et al.*, 1998a; Kanamaru *et al.*, 1999). It has been shown that divalent cations can bind directly to the 14-3-3 proteins and induce the conformational changes needed for the formation of a NR/14-3-3 complex (Athwal *et al.*, 1998b). NR degradation is triggered by phosphorylation and binding of 14-3-3 proteins (Kaiser and Huber, 1997).

### 2.2.2 NR-dependent NO-production in plants

NO was observed to be predominant compound evolved during a purged *in vivo* assay with soybean NR derived from accumulated nitrite (Harper, 1981). Experiments with <sup>15</sup>N-labelled nitrate as substrate for nitrate reduction showed that NO<sub>x</sub> is produced from <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> (Dean and Harper, 1986). Dean and Harper (1988) found that the constitutive NR (cNR, EC 1.6.6.2) of soybean converts nitrite to NO probably at the MO-pterin center and this soybean NRA was NAD(P)H-dependent and was cyanide sensitive. Because the cNR is unique to *leguminosae*, the NO production in plants was considered to be a phenomenon limited to this family. Wildt *et al.* (1997) reported that plant species other than *Leguminosae*, including sunflower, sugar cane, corn, rape, spruce, spinach and tobacco, emit NO gas under certain conditions. According to this last observation, high rates of NO production were detected *in vitro* after addition of corn NR into a solution containing NO<sub>2</sub><sup>-</sup> and NADH over a physiological pH range (Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000). The NO signal was completely eliminated by the addition of hemoglobin, a quencher of NO. These results suggest that inducible NR (iNR, EC 1.6.6.1), as well as the cNR, could have ability to produce NO. Rockel *et al.* (2002) showed that the NO generating capacity of NR *in vitro* could only account for a small part (< 1%) of the total NRA extracted.

NR-deficient mutants aid greatly to prove the role of NR in NO generation. Results obtained with boiled leaflets (Harper, 1981) and a mutant soybean line (NR1), which did not evolve NO, unlike WT plants (Nelson *et al.*, 1983; Ryan *et al.*, 1983; Dean and Harper, 1986, 1988) indicated that NR is a likely candidate for NO production. Recently, in the

green alga *Chlamydomonas reinhardtii*, using a NO-specific electrode and the fluorescence probe DAF-2DA, Sakihama *et al.* (2002) showed that nitrite induce NO generation in dark and this response was absent in a NR-lacking mutant (cc-2929).

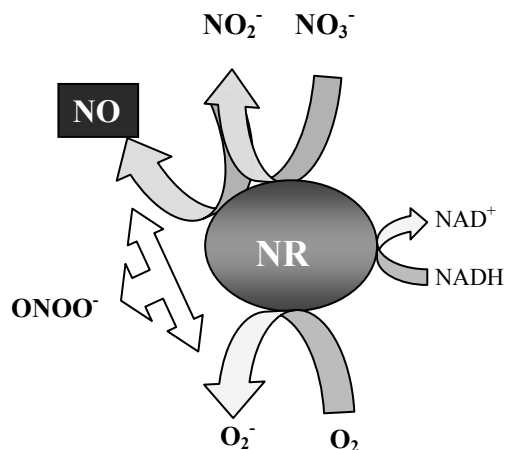
Several lines of evidence from both *in vitro* and *in vivo* assays have clearly indicated that NR is responsible for NO production in leaves. Obviously, accumulation of nitrite in the cells is the key event to reveal the condition where the NR-dependent NO production occurs. The first report on NO research in plant sciences was made by Klepper (1979), where he demonstrated the production of NO from herbicide-treated soybeans grown on nitrate as the nitrogen source. Klepper was able to evoke NO<sub>x</sub> emission by treating soybean plants with photosynthetic inhibitor herbicides (Klepper, 1978, 1979) or certain chemicals (Klepper, 1990, 1991) as well as under dark anaerobic conditions (Klepper, 1987, 1990). It was suggested that this emission was due to chemical reactions of accumulated nitrite with plant metabolites such as salicylate derivatives or the chemical decomposition of HNO<sub>2</sub>.

Using NiR deficient tobacco transformants, Morot-Gaudry-Talarmain *et al.* (2002) demonstrated that these plants, which accumulate nitrite, produced a much higher NO emission than the WT. On the other hand, high emissions of NO from plants have been observed in the dark under certain conditions (Klepper, 1979, 1990; Rockel *et al.*, 1996; Wildt *et al.*, 1997) or in the presence of photosynthetic electron inhibitors which interfere with the electron flow within chloroplasts and block nitrite reduction without affecting the reduction of nitrate. This differential leads to nitrite accumulation in the cell (Klepper, 1979; Mallick *et al.*, 1999).

### **2.2.3 Peroxynitrite -production by NR**

Barber and Kay (1996) reported that *Chlorella* NR produces O<sub>2</sub><sup>-</sup>, using molecular oxygen as an electron acceptor, when NADH is provided under aerobic conditions. The MO-pterin center of the enzyme is the site for the O<sub>2</sub><sup>-</sup> production (Ruoff and Lillo, 1990). O<sub>2</sub><sup>-</sup> production represented 0.5 % of the nitrate reduction activity. Yamasaki and Sakihama (2000) showed that under aerobic conditions, and with NAD(P)H, purified NR converts NO to ONOO<sup>-</sup> when nitrite is provided as the substrate for NR (Figure 6). ONOO<sup>-</sup> has been considered to be a major cytotoxic agent of active nitrogen species derived from NO (Squadrito and Pryor, 1998; Wink and Mitchell, 1998; Lipton *et al.*, 1993; Bolwell, 1999; Durner and Klessig, 1999). Sodium azide (an NR inhibitor), glutathione (an ONOO<sup>-</sup>

scavenger) and depletion of molecular oxygen all completely suppress the formation of  $\text{ONOO}^-$ , indicating that NR is capable of producing three types of toxic molecules ( $\text{NO}$ ,  $\text{O}_2^-$ ,  $\text{ONOO}^-$ ) when nitrite is provided as the substrate (Yamasaki and Sakihama, 2000).



**Figure 6:** Reactions catalysed by NR. NAD(P)H as electron donor can drive three different reactions: (i) the reduction of nitrate to nitrite (two electron transfer), (ii) the reduction of nitrite to NO (one electron transfer), and (iii) the reduction of oxygen to superoxide radical ( $\text{O}_2^-$ ; one electron transfer). (From Yamasaki and Sakihama, 2000, modified.)

### 2.3 Other enzymatic sources of NO

Other enzymes can also produce NO. Recently, a new plasma membrane-bound enzyme, nitrite: NO-reductase (NI-NOR) was discovered to be involved in NO formation from nitrite by plant roots (Stöhr *et al.*, 2001; Meyer and Stöhr, 2002). The plasma-membrane (PM) - associated NI-NOR may reduce the apoplastic nitrite produced by PM-NR *in vivo* and may play a role in nitrate signalling *via* NO formation. But this activity was not nitrite-dependent and was not affected by NR inhibitors.

Xanthine oxidoreductase (XOR: otherwise referred to as xanthine oxidase (XO; EC 1.1.3.22) or xanthine dehydrogenase (XDH; EC 1.1.1.204)), is also an enzyme recently shown to produce NO in humans (Harrison, 2002). XOR is a complex molybdo-flavoenzyme. The find that XOR can catalyze the reduction of nitrate and nitrite to NO, acting as a source of NO and  $\text{ONOO}^-$  (Godber *et al.*, 2000a), under anaerobic conditions, in the presence of either NADH or xanthine as reducing substrate is of special interest (Li *et al.*, 2001; Godber *et al.*, 2000b). XOR also reduces oxygen to  $\text{O}_2^-$  and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), key compounds responsible for oxidative cellular injury. It has also been reported that XO is capable of catalyzing the reduction of glyceryl trinitrate to NO under hypoxic conditions in the presence of NADH (Millar *et al.*, 1997, 1998; Zhang *et al.*, 1998).

## 2.4 *Non-enzymatic sources of NO*

In humans, NO was shown to be produced non-enzymatically from  $\text{NO}_2^-$  in the presence of a reductant, such as ascorbate. As this reaction requires the undissociated acid form of  $\text{NO}_2^-$  ( $\text{HNO}_2$ ,  $\text{pK} = 3.2$ ), chemical NO production is insignificant at physiological pH, but it might occur under acidic conditions in specific tissues and compartments (Weitzberg and Lundberg, 1998). Bethke *et al.* (2004) also demonstrated that NO can be synthesized non-enzymatically by the chemical reduction of  $\text{NO}_2^-$  in the apoplast of *Hordeum vulgare* aleurone layers. This NO production is accompanied by a loss of  $\text{NO}_2^-$  in the medium.

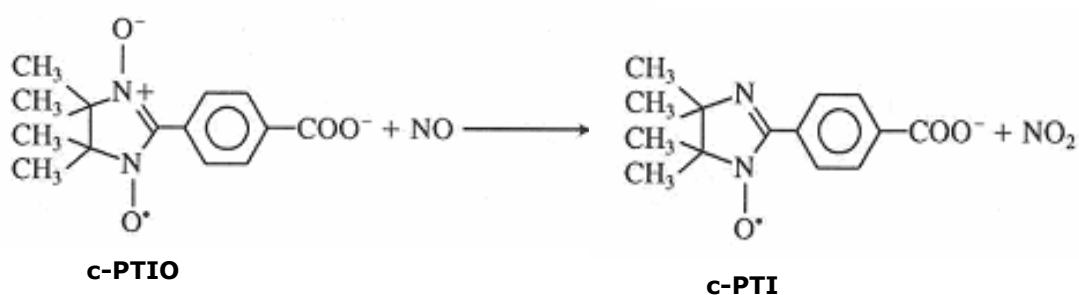
Light-mediated conversion of nitrogen dioxide to NO can be catalysed by carotenoids although this requires an acid pH and will only occur in selected compartments in the cells (Cooney *et al.*, 1994). The formation of nitrosating agents from the reaction of carotenoids with  $\text{NO}_2$  suggests that their ability to prevent nitrosative damage associated with  $\text{NO}_2$  exposure in plants may be limited in the absence of light.

## 3 **Methods of measuring NO**

### 3.1 *NO donors and NO scavengers*

Treatment of biological samples systems with NO gas is sometimes technically difficult. Therefore, chemicals that have the capacity to release NO (with or without enzymatic metabolism) have been widely employed as tools to investigate the role of NO in developmental or physiological processes in plants. NO application to plants *via* a NO-donor is a simple technique. A range of concentrations can be used and as NO released can be often measured, it is possible to investigate NO dose-responses. It is assumed that the physiological effects of NO donors are reproducible under different experimental conditions. Unfortunately, this is not always the case. NO-generating compounds differ in their chemical and biological properties. Effects that are independent of NO release or mediated by the release of alternative redox species of NO have been demonstrated for all classes of NO donors (Nedvetsky *et al.*, 2000). The rate of NO release from different NO donors can vary drastically with different experimental conditions.

Various NO donors have been employed and the most widely used is SNP (sodium nitroprusside) (Delledonne *et al.*, 1998, 2001; Clarke *et al.*, 2000; Pedroso *et al.*, 2000; Beligni *et al.*, 2001a), a compound that is in fact likely to generate  $\text{NO}^+$  (Stamler *et al.*, 1992).  $\text{NO}^+$  is a very reactive redox-related species of NO and rapidly reacts with buffer components, even with water (Koppenol, 1996). Therefore it is extremely difficult to predict the real concentration of NO produced by SNP in solutions. Other NO donors include (GSNO) S-nitrosoglutathione, a compound that does release NO but that may have other effects as well (Durner *et al.*, 1998), SNAP (S-nitroso-N-acetylpenicillamine; Durner *et al.*, 1998), RBS (Roussins's Black Salts; Clarke *et al.*, 2000), NOC-18 (Noritake *et al.*, 1996) and NOR-3 (Huang *et al.*, 2002). The principal disadvantages of exogenous application of an NO donor are due to the fact that the concentration of NO inside the plant tissues depends, among others, on certain chemical features such as kinetics of release from the donor, temperature, and reducing power, among others. Another disadvantage is the difficulty to determine whether the observed effects are merely pharmacological or indeed have physiological implications. In order to be confident that the effects of NO donors are due to NO release, and not due to the chemicals, it is useful to show that the effects of the donor can be abolished by application of a NO scavenger. The NO scavengers PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and c-PTIO (carboxy-PTIO) are commonly used. c-PTIO inactivates NO by oxidative transformation to  $\text{NO}_2$  (Akaike *et al.*, 1993; Figure 7). The reaction between NO and PTIO is believed to be stoichiometric.



**Figure 7:** Reaction between c-PTIO and NO.

The water-soluble c-PTIO reacts rapidly with NO to yield the corresponding imidazolinexyl (c-PTI) and free  $\text{NO}_2$  radical (from Pfeiffer *et al.*, 1997).

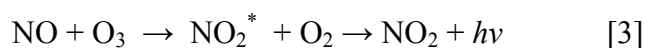
### 3.2 Demonstration and quantification of NO

Real-time measurement of NO in biological preparations is often very desirable. However, the instability of NO in aqueous solution and its high reactivity with various other species can cause difficulties for its measurement depending on the detection method employed. Different techniques and methodologies used in measuring NO are briefly summarised (Lamattina *et al.*, 2003).

#### ■ Direct measurements

A variety of methods have been used to monitor NO directly in the aqueous phase, notably using a NO-electrode. The electrochemical (amperometric) detection is reliable and sensitive. Oxidation of NO proceeds by a one electron transfer from NO to the anode, generating a nitrosonium cation ( $\text{NO}^+$ ), which is ultimately converted to nitrite. The oxidation rate of NO is directly proportional to NO concentration. Although this sensor offered a good detection limit (10 nM), it suffered from a very narrow linear range, irreproducibility, membrane fragility and high temperature dependence.

NO emission from plants could be also estimated by gas chromatography and mass spectrometry (Dean and Harper, 1986; Magalhaes *et al.*, 2000), laser photo-acoustic spectroscopy (Leshem and Pinchasov, 2000) and chemiluminescence detection (Wildt *et al.*, 1997; Morot-Gaudry-Talarmain *et al.*, 2002; Rockel *et al.*, 2002) where NO reacts with ozone to form nitrogen dioxide, a portion of which is in an excited state. As this  $\text{NO}_2$  species returns to ground state, light is emitted [3].



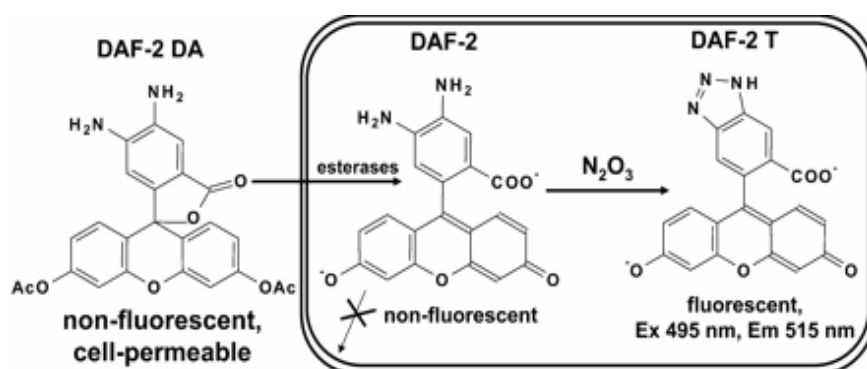
Gas phase chemiluminescence detection offers the best sensitivity and selectivity. However, it has some drawbacks such as bulky instrumentation, it is too costly for occasional quantitative analysis. However, as this technique has been used in this work, it will be described in more detail in the part “Materials and Methods”.

#### ■ Indirect measurements

The short and variable biological half-life of NO limits the study of its physiological effects by the above-described techniques. In spite of these difficulties, several indirect

methods for detecting NO have been developed. In aqueous solution, NO is rapidly converted to nitrate and nitrite. Spectrophotometric quantitation of nitrite using the Griess Reagent is a simple method. However, it does not measure nitrate. Therefore, the NADH-dependent enzyme NR is used to convert the nitrate to nitrite prior to quantitation using the Griess Reagent, thus providing for accurate determination of total NO produced. But major drawbacks to this method are that it is unable to detect changes in NO production in cells over time, and it is difficult to distinguish between the various sources of these species (*e.g.* nitrite derived from ONOO<sup>-</sup>). Most importantly, many plant cells possess NRA by which produces nitrite.

Fluorometric methods are very useful in real-time biological imaging of NO (Foissner *et al.*, 2000; Pedroso *et al.*, 2000; Desikan *et al.*, 2002; Neill *et al.*, 2002a). Several years ago, Kojima *et al.* (1998) developed fluorescence probes based on the fluorescein chromophore which are able to detect intra- as well as extra-cellular NO (Nakatsubo *et al.*, 1998). The probe mostly used up to now is 4,5-diaminofluorescein (DAF-2). DAF-2 which itself shows only low fluorescence, reacts in presence of oxygen with NO to form the highly fluorescent triazolofluorescein (DAF-2T). DAF-2 is membrane impermeable. To measure intracellular NO, cells can be loaded with the membrane permeable DAF-2 diacetate (DAF-2DA) which can be taken up by the cells and hydrolyzed by cytosolic esterases to form again the membrane-impermeable compound DAF-2 (Figure 8).



**Figure 8:** 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 N<sub>2</sub>O<sub>3</sub>, nitrosate DAF-2 to yield its highly fluorescent product DAF-2T (from Tarpey *et al.*, 2004).

DAF-2DA was used in a number of studies detecting intracellular NO mainly by confocal microscopy and also by fluorimeter. *In vitro*, studies have shown that DAF-2 does not react with stable oxidized forms of NO such as  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , nor with ROS such as  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$  and  $\text{ONOO}^-$ . The detection limit of NO by DAF-2 was 5 nM. The reliability of this method, however, was recently seriously questioned by Broillet *et al.* (2001) showing that, in the presence of NO, divalent cations like  $\text{Ca}^{2+}$  increase DAF-2 fluorescence. The  $\text{Ca}^{2+}$  sensitivity of DAF-2 thus makes it difficult to distinguish an intracellular  $\text{Ca}^{2+}$  increase from an increase in intracellular NO. But contrary to this report of divalent cation sensitivity and photoactivation of DAF-2, Suzuki *et al.* (2002) showed that with NO gas the reaction of DAF-2 and NO is completely independent of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at physiological concentration.  $\text{Ca}^{2+}$  enhances not the conversion of DAF-2 into its fluorescent product (DAF-2T), but the release of NO from NO donors. Another interesting recent finding indicated that DAF-2 fluorometric assays are quantitatively difficult to interpret in cells and in solution, when oxidants and NO are co-generated (Jourdeuil, 2002).

NO can also be measured by reaction with molecules such as hemoglobin (Delledonne *et al.*, 1998). This technique is based on the direct reaction between NO and the oxygenated ferrous form of hemoglobin ( $\text{HbO}_2$ ), which yields the ferric form, methemoglobin, and nitrate (Murphy and Noack, 1994) [4]. The disadvantage of the technique is that hemoglobin can also react with other molecules such as ROS that may be formed together with NO, and this assay might detect other nitrosyl groups in the sample.



NOS catalyses oxidation of the guanidine nitrogen of arginine, resulting in formation of NO and stoichiometric amounts of citrulline. Thus, the net potential of a tissue or cell extract to generate NO can be estimated by rates of formation of citrulline from arginine in the presence of saturating concentrations of NOS co-factors FAD, FMN, NADPH, tetrahydrobiopterin, calcium and calmodulin. On the other hand, arginine can also be converted to citrulline without the production of NO (Ninnemann and Maier, 1996). L-citrulline could also be produced by other enzymes, such as arginine aminase, argininosuccinate synthase and ornithine carbamoyl-transferase.



NO can also be detected as a free radical by electron paramagnetic resonance (EPR) spectroscopy which is used to quantify NO within tissues (Pagnussat *et al.*, 2002; Dordas *et al.*, 2003; Xu and Zhao, 2003). NO will react with “spin-trap” probes to form a stable adduct with characteristics EPR spectral properties that can then be monitored. EPR offers the advantage of continuous real-time monitoring of NO with a detection limit of 500 nM in solution, using hemoglobin spin trap. However, this detection limit is quite high in comparison with DAF-2DA (100 fold higher).

## 4 Functions of NO in plants

### 4.1 NO in the atmosphere

#### 4.1.1 Roles and principal sources of NO in the atmosphere

NO has been subject of immense interest to environmental scientists for many years, particularly due to its dual role in the catalytic formation and destruction of ozone (O<sub>3</sub>). In NO-rich air, such as those encountered in industrialized regions, reactive nitrogen oxides (NO<sub>x</sub> = NO + NO<sub>2</sub>) also play a key role in the chemistry of the lower atmosphere; NO is important for atmospheric radical balance and for generation of photo-oxidants (Wildt *et al.*, 1997).

Sources of NO<sub>x</sub> may be both anthropogenic and natural. NO<sub>x</sub> concentrations may range up to 100 ppb in the air over industrialized (fossil fuel combustion) areas (Wildt *et al.*, 1997). The release of NO from natural sources is mainly attributed to the activity of soil micro-organisms. An important turning point in NO research was the recognition of NO as a controversial intermediate in the bacterial denitrification processes (Williams *et al.*, 1992).

Paradoxically, few years ago, the vegetation has not been taken into an account as a natural source of NO in the atmosphere. Wildt *et al.* (1997) have clearly indicated that plant NO emissions should be considered as significant donors of atmospheric NO<sub>x</sub>. Indeed, NO<sub>x</sub> have been detected as natural emissions from vegetation (Hari *et al.*, 2003), although less than 0.1 % of the nitrogen taken up by plants is volatilised as NO. Recently, it has been suggested that global emissions from boreal coniferous forests might be comparable to those produced by world-wide industry and traffic (Hari *et al.*, 2003).

#### **4.1.2 Direct fixation of atmospheric NO by higher plants**

The NO<sub>x</sub> present in the atmosphere can be absorbed by plants with as little damage as possible. The question “could atmospheric NO<sub>x</sub> be a natural source of N for plants?” arose some years ago. The ability of vegetation to assimilate NO<sub>x</sub> from the atmosphere is well established. NO<sub>x</sub> uptake has been found to decrease when atmospheric NO<sub>x</sub> concentration decreases until a compensation point is reached (Wildt *et al.*, 1997; Hereid and Monson, 2001). The uptake of NO<sub>x</sub> by plant leaves reflects the interplay between the NO<sub>x</sub> concentration gradient between the atmosphere and the intercellular air spaces of the leaf and the stomatal conductance to NO<sub>x</sub> transport (Weber and Rennenberg, 1996). Employing <sup>15</sup>N tracer elements, it has been shown that a wide variety of higher plants are able to rapidly absorb NO<sub>x</sub> from the atmosphere and that patterns of plant assimilation of both NO<sub>2</sub> and NO are essentially identical (Eickriede *et al.*, 1995). It is possible that individuals capable of fixing NO<sub>x</sub> could be selected for a range of species, and genotypes with high rates of uptake could be of value as crops or for forestation in polluted areas to reduce tropospheric concentrations of NO<sub>x</sub>.

#### **4.2 NO during the plant life cycle: growth and development**

It has been known for some time that plants emit NO under normal growing conditions. NO and its related nitrogen dioxides have been reported as stimulators of seed germination (Grubisic and Konjevic, 1992). NO may also contribute to endogenous plant growth regulatory mechanisms in higher plants. Detrimental effects of NO on photosynthesis were reported many years ago and subsequently the effects of NO on plant growth were found to be concentration dependent (Hufton *et al.*, 1996; Leshem and Haramaty, 1996; Saviani *et al.*, 2002). It was shown that NO can reversibly suppress electron transport and ATP synthesis in chloroplasts (Takahashi and Yamasaki, 2002). 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. Morot-Gaudry-Talarmain *et al.* (2002) have shown that antisense-NiR tobacco plants accumulate nitrite, emit large amounts of NO and exhibit reduced growth. In the “pea disc expansion assay”, low NO levels promote foliage expansion growth, while higher levels are increasingly inhibitory (Leshem *et al.*, 1998). It has been also demonstrated that NO protects chlorophyll breakdown diminishing symptoms of chlorosis, necrosis and defoliation produced by the

methylviologen family of herbicides, causing ROS generation by accepting electrons from the chloroplastic electron chain (Laxalt *et al.*, 1997; Beligni and Lamattina, 1999a).

Recently, it has been reported that in *Atnos1* mutant plants (*Arabidopsis* mutant plants with impaired NO production) treated with 100  $\mu$ M SNP, greening, growth, and fertility were restored, indicating that these phenotypes were due to a deficiency in NO production caused by the *Atnos1* mutation (Guo *et al.*, 2003). The positive effects of NO on chlorophyll retention may reflect NO effects on iron availability. NO is closely related to iron metabolism (Murgia *et al.*, 2002), transport, and/or availability and, consequently, to chlorophyll biosynthesis and chloroplast development (Graziano *et al.*, 2002). Working with different plants, Wildt *et al.* (1997) demonstrated a light-dependent NO synthesis by plants which was positively correlated with the rate of CO<sub>2</sub> fixation in the light. This study drew a link between active photosynthesis, nitrogen assimilation and the accumulation of NO.

### **4.3 NO cross-talk with classical plant hormones**

Since NO can regulate processes related to plant growth and development, it has been termed a “non traditional” regulator of plant growth. Much evidence has been appearing lately about the cross talk between NO and some traditional phytohormones during adaptative responses to adverse conditions (Hausladen and Stamler, 1998; Beligni and Lamattina, 2001b), which will be briefly summarized.

#### **4.3.1 NO, ethylene and senescence**

NO is simultaneously emitted with ethylene in senescing pea foliage (Leshem and Haramaty, 1996; Haramaty and Leshem, 1997; Leshem, 2001). Nevertheless, some reports indicate that NO may have anti-senescence properties. The NO concentration appeared to be lower in mature fruits than in green fruits and in senescing compared with fresh ones (Leshem, 2001). Then, a decrease in NO production is linked with maturation and senescence and a concomitant increase of ethylene (Magalhaes *et al.*, 2000). NO extended the post-harvest life and delayed senescence (Leshem, 2001). Senescence is a process characterized by water loss and desiccation of plant tissues. Then, as NO has an antagonistic effect to ethylene during senescence process, NO was assumed to regulate

stomatal closure by modulating ion channels and  $\text{Ca}^{2+}$  levels in guard cells. In accordance with this suggestion, Hung and Kao (2003) have shown that NO counteracts the senescence of rice leaves induced by abscisic acid (ABA).

#### 4.3.2 NO, ABA and water deficit

In addition to promote leaf senescence, ABA can be accumulated in leaf tissue under drought stress, generating a loss of guard cell turgor that leads to stomatal closure, thus reducing transpirational water loss. Indeed, in *Pisum sativum*, in *Vicia faba* and in *Arabidopsis*, ABA induced an increase of endogenous NO levels in guard cells and other epidermal cells (Desikan *et al.*, 2002; Garcia-Mata and Lamattina, 2002; Neill *et al.*, 2002a) and it was shown that exogenous addition of NO to epidermal strips was sufficient to induce stomatal closure, through a  $\text{Ca}^{2+}$ -dependent process (Garcia-Mata and Lamattina, 2001; Desikan *et al.*, 2002; Neill *et al.*, 2002a). However, recent data attribute to NO a role for stomatal opening in *Vicia faba* (Sakihama *et al.*, 2003) contradicting these reported by Garcia-Mata and Lamattina (2001). Recently, it was shown that NO mediates the regulation of ion channels ( $\text{K}^+$  and  $\text{Cl}^-$  channels) in guard cells of *Vicia faba* through a subset of ABA-evoked signalling pathways (Garcia-Mata *et al.*, 2003).

It was suggested that ABA-mediated NO in guard cells is generated *via* a NOS-like activity (Neill *et al.*, 2002a; Guo *et al.*, 2003), and/or *via* NR (Desikan *et al.*, 2002; Garcia-Mata and Lamattina, 2002, 2003; Neill *et al.*, 2003). Nitrite was shown to induce NO accumulation in guard cells and stomatal closure, both events being abolished by NO scavengers (Desikan *et al.*, 2002; Neill *et al.*, 2003). Moreover, guard cells of the NR-deficient *Arabidopsis nia1*, *nia2* mutant did not synthesize NO nor did stomata close in response to nitrite or ABA (Desikan *et al.*, 2002). ABA did not induce NO synthesis in *Arabidopsis* suspension cultures (Tun *et al.*, 2001) which was interpreted as tissue specificity. Recently, Neill *et al.* (2002a) reported that nicotinamide, the antagonist of cyclic ADP ribose (cADPR) production, inhibits the effects of both ABA and NO on stomatal closure, suggesting that cADPR synthesis is part of the NO signalling pathway. Similarly to ABA accumulation, many environmental stresses commonly cause oxidative stress in plant, which results from over-production of reactive oxygen. Indeed,  $\text{H}_2\text{O}_2$  and NO have been identified to concert in regulating ABA-induced stomatal closure in various species (Neill *et al.*, 2002b; Desikan *et al.*, 2004). In support of this, Zhao *et al.* (2001)

have shown an interaction between ROS and NO in drought-induced ABA synthesis in root tips of wheat seedlings.

#### **4.3.3 NO, auxins and development of root architecture**

Auxin is the main hormone that regulates root architecture. A transient increase in NO concentration was shown to be required and to be part of the molecular events involved in adventitious root development of cucumber explants induced by indole acetic acid (Pagnussat *et al.*, 2002). Auxin-induced root growth and formation of lateral roots was also blocked by the NO scavenger c-PTIO (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004). Previous results reported by Gouvea *et al.* (1997) showed that NO-releasing substances induced root tip elongation of maize root segments in a dose-dependent manner, although in this case the effects of auxin were not reversed by an NO scavenger. A mitogen activated protein kinase (MAPK) signalling cascade could be activated during the adventitious rooting process induced by indole acetic acid in a NO-mediated pathway.

#### **4.3.4 NO, cytokinins, gibberellins and phytochrome**

Cytokinins lead to a rapid stimulation of NO release in cell cultures which may indicate a potential role for NO in cytokinin signal transduction (Scherer and Holk, 2000; Tun *et al.*, 2001). Cytokinins can also stimulate photomorphogenic responses, mainly those related with the de-etiolation process and pigment synthesis. Beligni and Lamattina (2000) have shown that NO promotes seed germination and de-etiolation, and reduces hypocotyl and internode elongation in *Arabidopsis* and lettuce seedlings grown in dark. Furthermore, NO is able to increase the chlorophyll level in wheat seedlings grown in the dark, but the NO effect is strongly potentiated by short-term light pulses. Then, the NO effect is similar to that of cytokinins and NO could take part in light-mediated signals, dependently and/or independently of photoreceptors. Beligni and Lamattina (2000) also demonstrated that NO donors are able to promote germination in the dark to the same extent as a gibberellin treatment or a 5-min pulse of white light. However, seeds were also able to germinate in the light, in the presence of the NO-scavenger c-PTIO, suggesting that light and NO can stimulate germination using the same or different pathways.

## 5 NO in plant pathology

Environmental stress is a major factor that limits plant productivity. Plants must continuously defend themselves against attack from bacteria, viruses, fungi, invertebrates, and even other plants. Because their immobility precludes escape, each plant cell possesses both preformed and an inducible defense capacity. Plants, which are unable to prevent pathogen regression, are called susceptible, and they frequently become systemically infected. This susceptibility may be caused by an inability of the plant either to recognize the pathogen or to establish defense responses. In contrast, plants capable of restricting pathogen replication and/or movement to the initial site of infection are termed resistant. One of the most powerful weapons in plant's arsenal against pathogen attack is the hypersensitive response (HR), which could be considered as a programmed cell death (PCD) (Jabs *et al.*, 1997). The HR is characterised by rapid, localised cell death at the site of infection (Lamb and Dixon, 1997). This HR plays a central role in disease resistance (Heath, 2000). It is associated with the induction of defense-related genes that restrict pathogen growth, either indirectly, by helping to reinforce plant cell walls, or directly, by providing antimicrobial enzymes and toxic secondary metabolites, such as phytoalexins, which kill pathogens. A local HR is often associated with the onset of systemic acquired resistance (SAR) in distal plant tissues (Enyedi *et al.*, 1992; Ryals *et al.*, 1996). As a result, the entire plant is more resistant to a secondary infection. The HR is part of a complex suite of active defense responses induced *via* a range of molecular communications between plant and pathogen and requires elaborate host cell signalling mechanisms.

Although the effects of both ROS ( $H_2O_2$ ) and NO on plant physiology and development have been the subject of investigation for several years, it is only relatively recently that their role as signalling molecules during abiotic or biotic stresses have been recognized (Neill *et al.*, 2002b).

### 5.1 ROS and plant stress responses

Although ROS are formed in normal cell metabolism and their production is a common cellular event, oxidative damage is often associated with plant stress (Dat *et al.*, 2000). The most reactive of all ROS is the hydroxyl radical that is formed from  $H_2O_2$  by the so-called Haber-Weiss or Fenton reaction by using metal catalysts (Wojtaszek, 1997). It is well

known that the PCD occurring during the HR is preceded by an oxidative burst that is mainly attributable to the activation of several enzymatic systems involved in ROS generation. The principal production of ROS is catalyzed by a plasma-membrane-located NADPH oxidase. This is supported by the recent identification of genes in *Arabidopsis* that are homologous to the large subunit of human NADPH oxidase (Bolwell *et al.*, 2002). Chemical inhibitors of NADPH oxidase, such as diphenylene iodonium (DPI), have been shown to block or severely reduce ROS production upon biotic and abiotic stresses (Allan and Fluhr, 1997; Orozco-Cardenas and Ryan, 1999). Other different enzymatic sources of H<sub>2</sub>O<sub>2</sub> have been identified, including pH-dependent cell wall peroxidase, amine oxidase, oxalate oxidase and flavin-containing oxidases including xanthine oxidoreductase (Bolwell and Wojtaszek, 1997; Bolwell *et al.*, 2002). The produced ROS can act as an antibiotic toward the pathogen and reinforce the cell wall by catalyzing cross-linking of cell wall proteins through a peroxidase-dependent reaction, rendering the wall less digestible by microbial enzymes.

The production of ROS as a by-product of chloroplastic, mitochondrial, peroxisomal and glyoxysomal redox systems has long been recognised. ROS are useful as signalling molecules in plant host defense but on the other hand they cause cellular damage, if not produced in a controlled manner. Therefore, plants possess a battery of antioxidant mechanisms by which ROS are removed from the cell (Noctor and Foyer, 1998). The main ones are SODs, a family of metalloenzymes catalyzing the dismutation of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub> (Bowler *et al.*, 1994), catalases, which are heme proteins that dismutate H<sub>2</sub>O<sub>2</sub> to oxygen and water, and the enzymes and metabolites of the ascorbate-glutathione cycle that are involved in the removal of H<sub>2</sub>O<sub>2</sub>, notably ascorbate peroxidases which reduce H<sub>2</sub>O<sub>2</sub> to water by utilising ascorbate as specific electron donor (Noctor and Foyer, 1998). Thus, a critical balance between the production and distribution of ROS determines the fate of the cell.

In plants, ROS are generated in response to both abiotic and biotic stresses such as drought, exposure to ultraviolet light and ozone, extremes of temperature, light intensities and pathogen challenge (Dat *et al.*, 2000), whilst exogenous H<sub>2</sub>O<sub>2</sub> can induce tolerance to chilling, high temperatures, and biotic stress (Van Camp *et al.*, 1998). Several excellent reviews on the role of ROS during plant-pathogen interactions have been published so far (Lamb and Dixon, 1997; Bolwell, 1999; Dat *et al.*, 2000). These ROS, and in particular

H<sub>2</sub>O<sub>2</sub>, are able to cross cellular membranes and serve as diffusible signalling molecules for the induction of defense-related genes (Levine *et al.*, 1994; Jabs *et al.*, 1997; Desikan *et al.*, 1998) and for the initiation of plant cell death (Levine *et al.*, 1994; Alvarez *et al.*, 1998; Desikan *et al.*, 1998). Generation of ROS in plants can be detected within minutes of elicitor or microbial treatment (Apostol *et al.*, 1989; Low and Merida, 1996; Draper, 1997). The production of ROS occurs in two distinct phases: a weak initial, non-specific, phase follows within minutes of pathogen addition, and a secondary phase, dependent on recognition of incompatible pathogens by the host begins 1 to 3 h after the initial burst (Baker *et al.*, 1993; Levine *et al.*, 1994; Baker and Orlandi, 1995). This two-phase kinetics of ROS production is typical of incompatible plant-pathogen interactions that are characterized by the HR. In some plant-pathogen interactions, the oxidative burst in the induction of cell death and pathogen killing seems to be necessary, but not sufficient (Levine *et al.*, 1994; Glazener *et al.*, 1996; Jabs *et al.*, 1997; Dangl, 1998; Dorey *et al.*, 1999). Then, it is clear that plant cells require other signal molecules to activate HR in physiological conditions. Apart from the oxidative burst, HR is characterized by other metabolic disturbances, such as ion flux across the plasma membrane (Ca<sup>2+</sup> and H<sup>+</sup> influx and K<sup>+</sup>, Cl<sup>-</sup> efflux) as well as changes in pH and plasma membrane depolarisation (Heath, 2000), and by NO production, which will be shown below.

## **5.2 NO and stress**

### **5.2.1 Production of NO during HR**

Pathogen attacks may cause very localised NO production and thus localised high NO concentrations. This NO appears as a second messenger in plant pathogen resistance (Pfeiffer *et al.*, 1994; Delledonne *et al.*, 1998; Durner and Klessig, 1999; Bolwell 1999; Clarke *et al.*, 2000). 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* suspension culture when present at concentrations similar to those generated following challenge by avirulent pathogens (Clarke *et al.*, 2000). Saviani *et al.* (2002) showed that treatment of citrus cultures with NO induced cell death bearing the characteristics of PCD.



Soybean and *Arabidopsis* cell suspensions inoculated with *Pseudomonas syringae* produce NO with a pattern similar to H<sub>2</sub>O<sub>2</sub> 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. NOS activity was detected in this plant-pathogen interaction (Delledonne *et al.*, 1998). Moreover, tobacco epidermal peels treated with an elicitor responded with NO generation within minutes (Foissner *et al.*, 2000). With immunoblot analysis using antibodies against rabbit brain NOS, it has been found that tobacco plants resistant to infection by *Ralstonia solanacearum* exhibited elevated levels of NOS (Huang and Knopp, 1998). In accordance, an increase in constitutive NOS activity has been detected after infection of resistant, but not susceptible, tobacco with tobacco mosaic virus (TMV) (Durner *et al.*, 1998). Recently, Chandok *et al.*, (2003) demonstrated that the pathogen-inducible NOS (iNOS) in plants is a variant form of the P protein of GDC. Addition of a membrane permeable NO scavenger or NOS inhibitors completely blocked NO-dependent cell death and defense gene activation in tobacco and soybean (Delledonne *et al.*, 1998; Durner *et al.*, 1998, Foissner *et al.*, 2000), showing that NO plays a role in HR development.

PCD occurring as a result of mechanical stress may also involve NO. In *Kalanchoe daigremontiana*, centrifugation of leaves and callus induced NO generation and subsequent DNA fragmentation and cell death (Pedroso and Durzan, 2000; Pedroso *et al.*, 2000). Decreased NO synthesis and PCD in the presence of a NOS inhibitors suggested the involvement of a NOS-like enzyme in this species. Similar results were found with mechanical stress of *Arabidopsis* tissues (Garces *et al.*, 2001).

## **5.2.2 Concerted action of ROS and NO in plant defense responses**

### *5.2.2.1 Synergism between NO and ROS?*

There is now compelling evidence that H<sub>2</sub>O<sub>2</sub> and NO both function as signalling molecules in plants (Bolwell, 1999; Durner and Klessig, 1999; Dat *et al.*, 2000; Neill *et al.*, 2002b). In mammalian macrophages, ROS collaborate with NO to execute bacterial pathogens (Nathan, 1995). In plants, it has also been reported that NO and ROS have important roles in the activation of defense responses against pathogen attacks and cooperate

synergistically in the induction of HR (Dangl *et al.*, 1996; Wojtaszek, 1997; Delledonne *et al.*, 1998; Bolwell, 1999; McDowell and Dangl, 2000; de Pinto *et al.*, 2002; Lum *et al.*, 2002). However, Tada *et al.* (2004) suggested that NO and  $O_2^-$  might not be essential mediators for the initiation of hypersensitive cell death induced by a fungus in oat, although they would be important regulators for apoptotic cell death in the adjacent cells.

Using cell suspension cultures of *Arabidopsis thaliana*, Clarke *et al.* (2000) have found that NO acts independently of ROS to initiate PCD with regards to the different kinetics of NO and ROS production and that NO-induced cell death does not result from the production of the highly reactive free radical ONOO<sup>-</sup> formed by the reaction with  $O_2^-$  and NO. Nevertheless, NO also seems to act independently from ROS in the induction of various defense genes including pathogenesis-related proteins and enzymes of the phenylpropanoid metabolism that are involved in the production of lignin, antibiotics and the secondary signal salicylic acid (Noritake *et al.*, 1996; Delledonne *et al.*, 1998).

Recently, a model has been proposed in which the interaction between ROS and NO can determine whether or not PCD is the outcome. The NO:  $O_2^-$  ratio should determine PCD (Delledonne *et al.*, 2001). A correlation between  $H_2O_2$ , NO and antioxidant levels has also been demonstrated recently by de Pinto *et al.* (2002).

#### 5.2.2.2 *Combination of NO with ROS: protective or toxic effect?*

The effects of NO on different types of cells have been shown to be either protective or toxic, depending on its concentration and on the situation. As mentioned above, Delledonne *et al.*, (1998) reported that NO acted synergistically with ROS to potentiate the HR, while NO alone had little effect on cell viability (de Pinto *et al.*, 2002). NO-mediated toxicity is mainly generated by reaction with the  $O_2^-$  anion leading to the formation of ONOO<sup>-</sup> which may be also involved in killing cells of the pathogen and of the host alike, at least in high concentrations, as in the mammalian inflammatory response (Squadrito and Pryor, 1998; Bolwell, 1999). However, it was also observed in soybean cell suspensions, that ONOO<sup>-</sup> is not an essential mediator of NO/ROS-induced cell death (Delledonne *et al.*, 2001), but is expected to have important physiological and signalling functions in the HR, since the ONOO<sup>-</sup> donor SIN-1 was found to induce accumulation of the transcript encoding PR-1 in tobacco leaves (Durner *et al.*, 1998). Urate, a scavenger of ONOO<sup>-</sup>, significantly reduced lesion formation caused by an exogenous ONOO<sup>-</sup>-generating system,

by direct application of ONOO<sup>-</sup> or by an avirulent pathogen in *Arabidopsis* leaves (Alamillo and Garcia-Olmedo, 2001). Thus, the inability of ONOO<sup>-</sup> to induce cell death might be specific to soybean and requires further clarification.

The ability of NO to scavenge ROS had been first reported in animals (Stamler, 1994). The potential cytoprotection is based on NO's ability to regulate the level and toxicity of ROS. The presence of NO can attenuate the Fenton oxidative damage preventing the formation of oxidants by scavenging either iron or O<sub>2</sub><sup>-</sup> and thus limiting hydroxyl radical formation (Wink *et al.*, 1995; Beligni and Lamattina, 2002b). The NO molecule itself possesses antioxidant properties. Several studies have demonstrated that NO can act as a chain-breaking antioxidant arresting lipid peroxidative reactions (Hogg *et al.*, 1993; Rubbo *et al.*, 1994; Kelley *et al.*, 1999). The cytoprotective effects of NO in plants were reported under strong oxidative conditions during both biotic and abiotic stresses, even under photo-oxidative situations (Laxalt *et al.*, 1997; Beligni and Lamattina, 1999a, b, c, 2002b; Garcia-Mata and Lamattina, 2001; Hung *et al.*, 2002). Cytoprotection against the oxidative burst was observed at different levels of organization such as cell culture, tissue, organ, and at the whole plant level, and exerted on all tested macromolecules: DNA, RNA, protein, chlorophyll and lipids (Beligni and Lamattina, 1999c, 2002b). Then, there is an apparent contradiction between the situation in which NO appears to counteract ROS actions and those in which it acts in the same direction or synergistically with ROS to potentiate cell death during the HR.

### **5.2.3 Signal transduction through NO and its downstream messengers**

#### *5.2.3.1 Cyclic guanosine monophosphate (cGMP) dependent pathways of NO action*

Despite the multiplicity of potential targets, only few proteins have as yet been demonstrated to be regulated by interaction with NO. In mammalian cells, many of the cellular effects of NO appear to be mediated by cGMP (Stamler, 1994). In plants, the NO treatment of spruce needles has been shown to cause transient increases in cGMP levels (Pfeiffer *et al.*, 1994). In animals, NO activates cGMP production from GTP *via* interaction with a soluble form of GC (Wendehenne *et al.*, 2001). As NO is reasonably permeant, it reacts directly with the iron in the heme moiety of GC, inducing a conformational change that results in enzyme activation. It was shown that NO signalling in tobacco or in *Arabidopsis* cells required cGMP synthesis but this second messenger was

not sufficient for the NO-induced cell death (Durner *et al.*, 1998; Clarke *et al.*, 2000). In NO-treated soybean cell suspensions (Durner *et al.*, 1998), cGMP was also involved in the induction of transcripts encoding phenylalanine ammonia lyase (PAL), the first enzyme of the phenylpropanoid biosynthesis pathway. Furthermore, the use of NOS inhibitors caused a marked reduction in the expression of chalcone synthase (Modolo *et al.*, 2002), the first enzyme of the branch specific for flavonoids and phytoalexins (Dixon and Paiva, 1995). Indeed, treatment of potato tuber tissues with the NO donor, NOC-18, provoked an accumulation of the phytoalexin rishitin, an endogenous antibiotic compound (Noritake *et al.*, 1996).

Another mode of action for NO and cGMP is to stimulate the synthesis of cADPR, an agent that mobilizes  $Ca^{2+}$  and thereby serves as a further downstream messenger of NO (Denninger and Marletta, 1999). In tobacco cells, cADPR was able to mimic NO induction of PR-1 and PAL gene expression and the cADPR effects were inhibited by ruthenium red (which inhibits  $Ca^{2+}$  release stores), indicating its calcium dependence (Durner *et al.*, 1998). Moreover, a cADPR antagonist suppressed NO induction of PR-1; however, this effect was incomplete, indicating that NO activation of defense responses may occur through more than one pathway (Klessig *et al.*, 2000). Although the involvement of cGMP in several plant signal transduction pathways has been demonstrated (Bowler *et al.*, 1994), it remains to be determined whether or not NO is the physiological activator of plant GC, because it should be noted that neither GC nor the phosphodiesterase responsible for cGMP degradation have been cloned from plants.

In addition, one potential intracellular target for NO is the interaction with MAPK signalling pathways. MAPK were recently found to be activated by NO in both *Arabidopsis* (Clarke *et al.*, 2000) and tobacco (Kumar and Klessig, 2000) and in this last case, MAPK in tobacco could also be activated by others signals such salicylic acid and  $H_2O_2$ . But the role of MAPK in the induction of genes involved in defense has yet to be investigated.

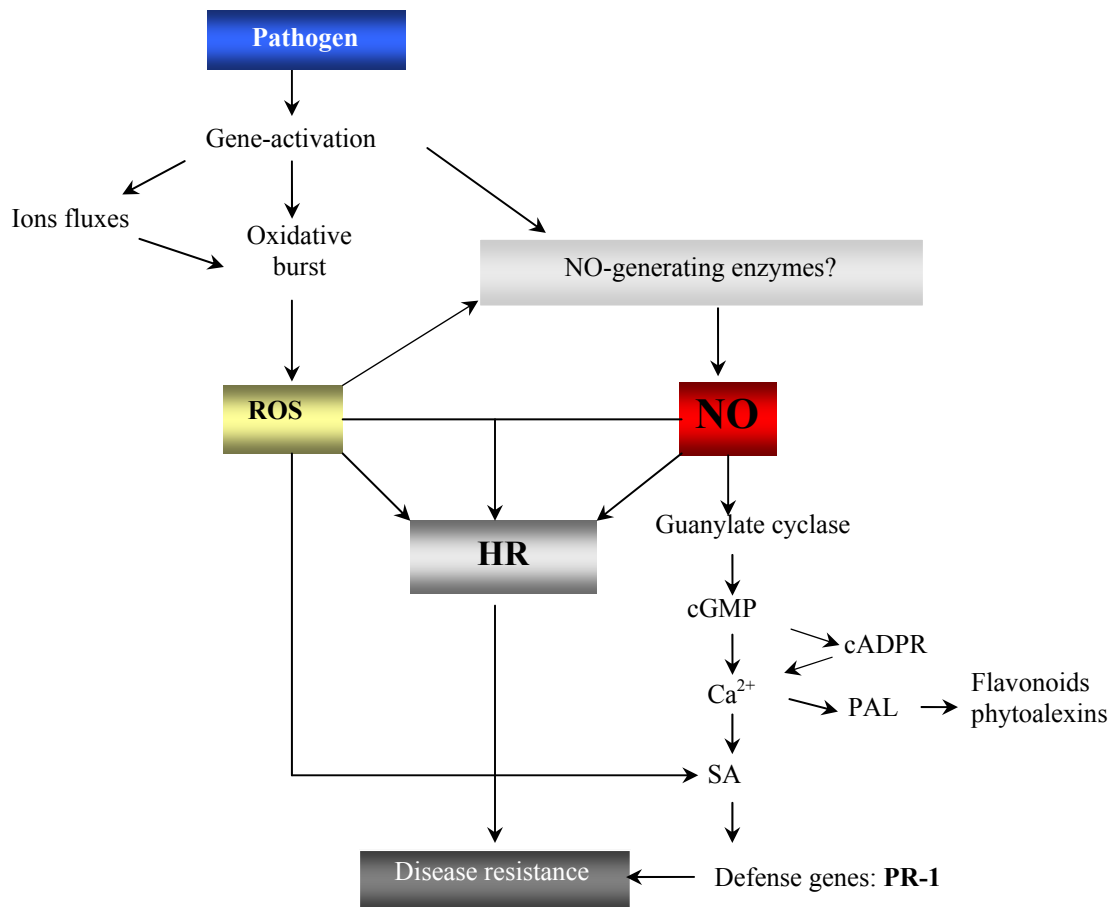
#### 5.2.3.2 NO and Salicylic acid

Recently, much attention has been addressed to elucidate the role of salicylic acid (SA) in plant disease resistance against pathogens (Durner *et al.*, 1997). Delledonne *et al.* (1998) showed that NO synergizes not only with ROS, but also with an endogenous factor that is

dependent on salicylic acid and potentiates the overall defense response (Shirasu *et al.*, 1997). A large body of evidence now indicates that SA, produced from the shikimate-phenylpropanoid pathway, is considered as a signalling molecule in the pathways leading to local and systemic disease resistance in plants (Ryals *et al.*, 1996; Leon *et al.*, 1995; Dangl *et al.*, 1996). As glutathione is a major metabolite in the phloem, where the SAR signal is transmitted, it might be hypothesized that excess NO produced during the HR binds to glutathione in which form it could serve as a long-distance SAR signal (Durner *et al.*, 1999).

NO as well as ROS have been shown to stimulate the accumulation of SA, and SA again induces the production of ROS, such as H<sub>2</sub>O<sub>2</sub> (Durner and Klessig, 1999). Thus, these signals appear to be self-amplifying. Both ROS and SA have been shown to synergize with NO to enhance host cell death in soybean cell suspensions (Delledonne *et al.*, 1998). SA and NO may work synergistically to transduce the defense signal by targeting the same effector proteins and/or their genes (Klessig *et al.*, 2000). NO treatment of tobacco leaves induced a significant increase in endogenous SA and this increase was required for expression of the PR-1 gene (Malamy *et al.*, 1990; Klessig and Malamy, 1994; Durner *et al.*, 1998). Some PR proteins (e.g. chitinase, and B-1,3-glucanase) are hydrolytic enzymes capable of lysing cell walls and cell membranes of invading pathogens, whereas others (e.g. peroxidase) are able to fortify plant cell walls. The ability of NO to up-regulate PAL mRNA levels (Durner *et al.*, 1998) means that this gas may be involved in the plant defense mechanisms by both SA-dependent and -independent pathways (Chamnongpol *et al.*, 1998; Durner *et al.*, 1998; Van Camp *et al.*, 1998). Similar to SA, jasmonic acid (JA) also is believed to play an important role in influencing plant resistance to pathogens and other stress factors. However, the relationship between SA and JA in mediating defense responses are clearly complex and poorly understood. There are examples of antagonistic as well as synergistic interactions to biotic and abiotic stresses (Rao *et al.*, 2000; Glazebrook, 2001). Recently, it has been reported that NO was induced by wounding and influenced JA signalling in *Arabidopsis thaliana* (Huang *et al.*, 2004).

However, in spite of much progress during the last decade, many details in the relationship between NO, SA and ROS in the activation of defense genes and/or induction of host cell death are currently not understood. The role of NO in plant defense against pathogens is summarized in Figure 9.



**Figure 9:** NO mediated signalling pathway in plant defense against pathogens; hypothetical model. Abbreviations are as follows:  $\text{Ca}^{2+}$ : calcium cADPR: cyclic ADP ribose, cGMP: cyclic GMP, HR: Hypersensitive Response, NO: nitric oxide, PAL: phenylalanine ammonia lyase, PR: pathogenesis-related proteins, ROS: Reactive Oxygen Species, SA: salicylic acid.

## 6 Aims of this thesis

As pointed out above, the potential role and production of NO in plants has gained increasing attention during the last decade. There are the two major lines of interest for plant science:

- A- How is NO synthesized in plants and how is NO synthesis controlled?
- B- How does NO function?

**This thesis is mainly devoted to investigate the reactions which potentially contribute to NO production by plants, and to quantify this NO production under normal and stress conditions.**

For measuring NO production from plants in a quantitative way, the highly sensitive on-line detection of NO by chemiluminescence was used. NO emission into purified air was followed in leaves, cell suspensions and purified enzyme solutions.

For elucidating the role of NR in NO production, we used various tobacco mutants and transformants over- or underexpressing nitrate or nitrite reductase and/or tobacco plants or cell cultures that were made free of NR and further free of MoCo enzymes by other means. The potential source(s) and emission of NO were studied in two situations:

- A- Normal environmental events such as light/dark, air/nitrogen or N-nutrition regimes
- B- Biotic stress, particularly in response to the fungal elicitor *Phytophthora cryptogea* (cryptogein) which is known to provoke an HR in tobacco (Ricci *et al.*, 1989). The potential role of NO as an intermediary signal in plant-pathogen interactions was also investigated.

## B RESULTS

### CHAPTER 1

#### NO production in simple systems

As already pointed out, in aqueous solutions, NO can rapidly react with oxygen and its derivatives to form other potentially toxic radicals. In biological systems, its hydrophobicity permits NO to readily permeate cell membranes, but the potentially short half-life makes it extremely difficult to predict or measure NO concentrations in living material. In medical and animal research, NO production has been predominantly estimated by the detection of accumulating NO oxidation products. These methods usually suffer from the inability to resolve temporal changes in NO production. In plants, they cannot be used anyway, since the major oxidation products of NO, nitrite and nitrate, are normal metabolites occurring (occasionally in high concentrations) in virtually any plant tissue. Therefore, we have used the high-sensitivity chemiluminescence NO-detection for a real-time NO measurement in biological samples.

Prior to measurement of NO in plants, we have examined the power and the limits of the method using simple systems, like solutions flushed with NO gas, solutions containing chemical NO-donors or NO-generating solutions of purified NR.

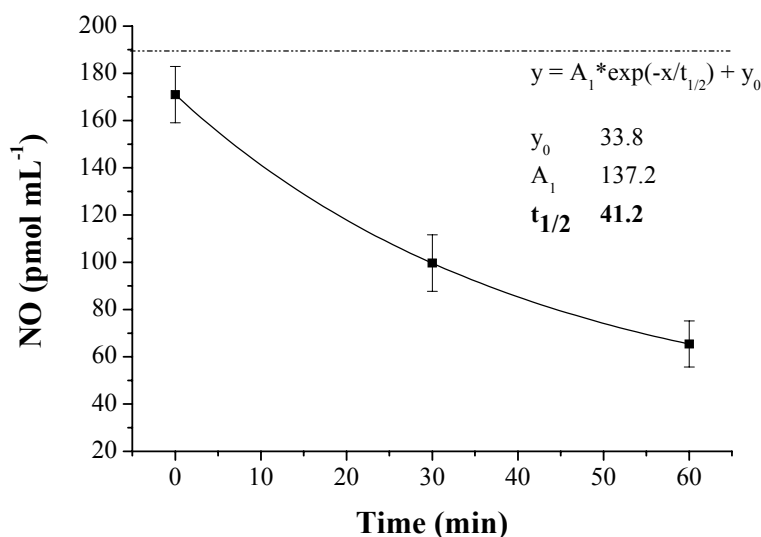
#### 1.1 Detection of NO released from solutions flushed with NO

NO is an uncharged gaseous free-radical with moderate water solubility: the Henry-Coefficient is 1.9 mM in pure water at atmospheric pressure. The physical half-life of NO in solution depends on its concentration. At the low concentrations (nanomolar) of NO to be expected in biological system, the physical half life in pure water is in the range of hours (Henry *et al.*, 1997). However, living cells contain and produce many compounds that react rapidly with NO, *e.g.* ROS, sulfhydryl groups and heme-compounds. Therefore the half-life of NO *in vivo* may be in the range of a few seconds (Lancaster, 1997) (also compare 1.1 in “Introduction”).



### 1.1.1 Stability of NO in buffer solution

For examining the physical half-life of NO in frequently used biological buffers, a solution of MES-KOH (50 mM, pH 5.5) was flushed with NO (100 ppm in nitrogen) for 15 min. According to the Henry coefficient (1.9 mM at atmospheric pressure), 1 mL of this solution contained 190 pmoles NO. Here, 2 mL of the above NO equilibrium solution were pulled into a syringe already containing 2 mL of air-saturated buffer. The syringe was immediately closed and left on the lab bench for up to one hour under gentle shaking, in order to allow NO to react with the dissolved oxygen. At  $T = 0$ ,  $T = 30$  min or  $T = 1$  h, the 4 mL aliquots were injected into the measuring cuvette under continuous stirring, under a stream of nitrogen. The time-course of the NO emission into the headspace from the solution was continuously followed (compare Figure 11) until the signal had again reached the baseline. Integration of the emission curves gives the total amount of NO released. If NO would react during incubation, the integral of the emission curve should become smaller. The initial amount of NO emitted from the solution was almost identical with the theoretical NO content derived from the Henry coefficient and decreased only slowly (Figure 10). The half-life of NO in MES-buffer pH 5.5 (calculated from the curve by first order exponential kinetic (Origin, OriginLab, Northampton, USA)) was 41.2 min.

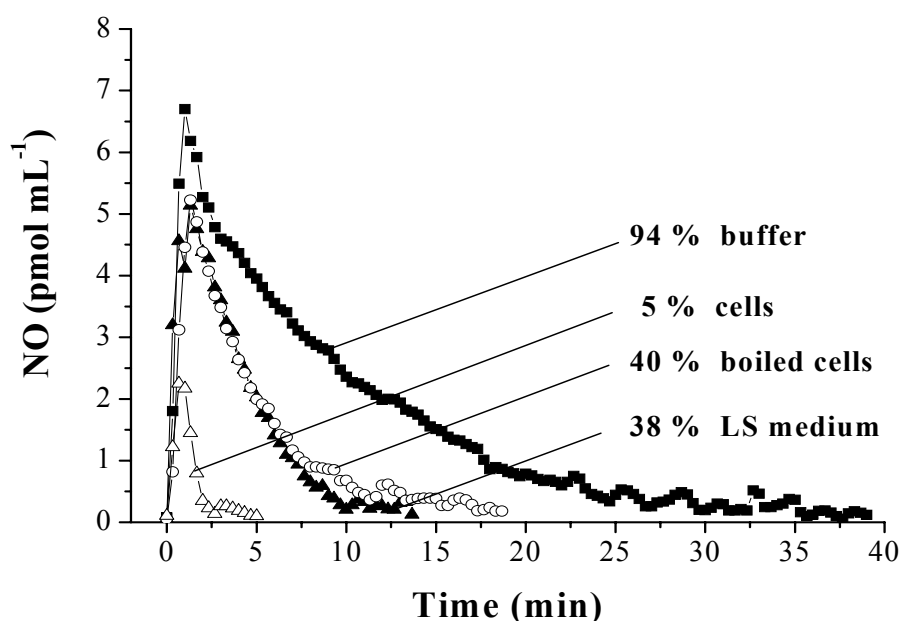


**Figure 10:** Determination of the physical half-life of NO in MES-buffer (50 mM pH 5.5), half-saturated with air.

The dashed line gives the theoretical initial NO concentration (pmol mL<sup>-1</sup>) derived from the Henry coefficient. The half-life was calculated using first order exponential kinetic analysis. Each point is a mean from four measurements. The bars describe the standard deviation.

### 1.1.2 NO-quenching by cell suspensions

For an initial check of the stability of NO in cell suspensions, slightly modified conditions were used. A solution with a defined amount of NO was prepared as before. Aliquots (2 mL) of NO solution were rapidly injected into a vigorously stirred buffer solution (1 mL) in the headspace cuvette, which was flushed with purified air. Expectedly, after injection the NO rapidly escaped from the stirred solution into the gas phase, where it was measured. At 22°C, it took about 30 min until NO emission from the solution came to an end (Figure 11). Integration of the emission curve revealed again that the recovery of the injected NO was almost complete (94 %). However, when 2 mL of the dissolved-NO solution was injected into a stirred tobacco cell suspension, only 5 % of the injected NO could be recovered, depending on stirring velocity. Obviously a large fraction of the NO had rapidly reacted in the cells. When the cells were killed by brief boiling prior to the injection of NO, still 40 % of the dissolved NO was emitted from the stirred solution (Figure 11).

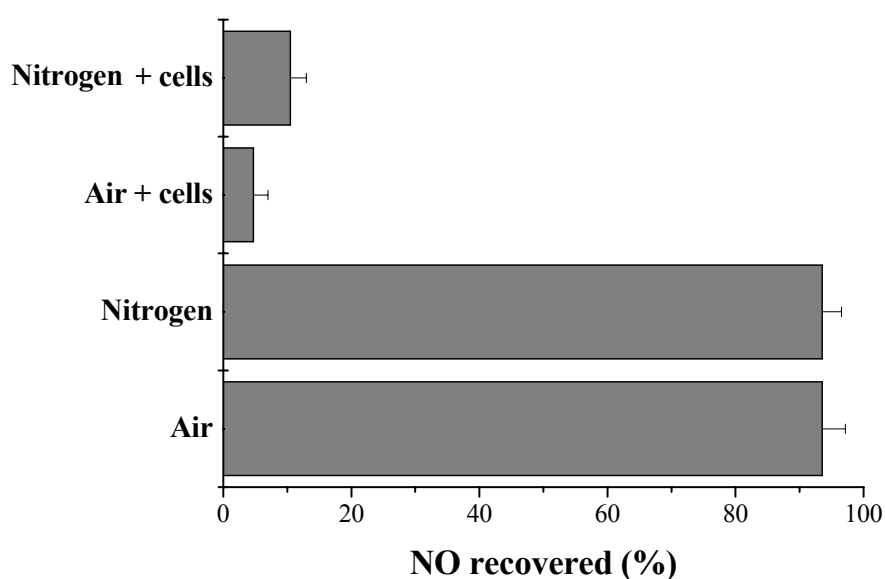


**Figure 11:** Effect of the quenching by cells, in air, of NO recovery from a defined source of NO.

Two mL of the solution, prepared as before, were injected into 1 mL of buffer solution (■), into 1 mL LS medium (▲), into 1 mL of a tobacco cell suspension (△), or into 1 mL of a boiled cell suspension (○) in a small Petri dish placed in a glass cuvette, mounted on a magnetic stirrer. Numbers at the curves give the total amount of NO (obtained by integrating the area under the emission curve) as percentage of the theoretically added NO. The data represent the means of five replicate experiments.

The same amount of NO was emitted when injected into the complex LS-medium (1 mL) used for the cell suspensions, but without cells. Obviously, a large part of the NO scavenging capacity of the cell suspensions was due to the medium itself. The experiment also demonstrates that living cells scavenge much more NO than boiled cells. Scavenging is therefore property of living cells or enzymes.

In order to check for an involvement of ROS in NO quenching, similar experiments were conducted under a stream of nitrogen gas. In pure buffer solution, up to 95 % of the added NO was again released into the gas stream in nitrogen (Figure 12), indicating that NO stability was not much affected by the presence of oxygen. Interestingly, when NO was injected into a cell suspension under a stream of nitrogen, about 10 % of the injected NO was released into the gas phase (Figure 12). Thus, while aerobic cells trapped about 95 % of the added NO, anoxic cells trapped only 90 %.



**Figure 12:** Effect of oxygen on the recovery (emission) of NO from solution or a cell suspension. NO emission was followed after injection of NO-solution (2 mL) into buffer (1 mL; 50 mM MES-KOH, pH 5.5) or into a cell suspension (1 mL) under air or nitrogen. Numbers give the amount of NO (obtained by integrating the area of the emission curve) as percentage of the theoretically added NO. The data represent the mean ( $\pm$  SD) of five replicate experiments.

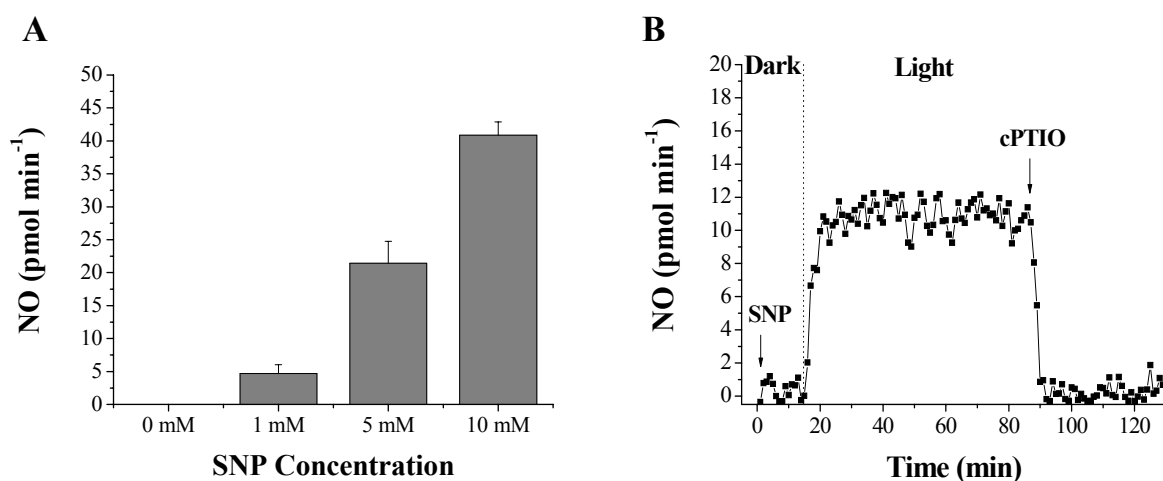
## 1.2 NO-emission from a NO donor: sodium nitroprusside

The above attempts (1.1) to quantify NO scavenging by solutions and cell suspensions do not completely mimic the natural situation, as a relatively high amount of NO is added at once, whereas normally, NO would be produced continuously. We therefore used SNP (sodium nitroprusside) as a source to continuously produce NO in solution. Although SNP is one of the most popular NO donors used in many studies, the exact mechanism of the chemical reactions leading to NO release is not completely understood (compare 3.1 in “Introduction”). The main NO-releasing form appears to be the nitrosyl cation ( $\text{NO}^+$ ), which has chemical properties different from those of the NO radical. NO production by SNP is heavily affected by thiols, transient metals, oxygen concentration or light (Feelisch, 1998).

### 1.2.1 Detection of NO by the decomposition of SNP

First, NO release into the gaseous phase above a 1 mL buffer solution (HEPES-KOH, 50 mM, pH 6.5) was measured with different SNP-concentrations. As shown in Figure 13A, the rate of NO-emission at steady state was linearly correlated with the SNP concentration, 1-10 mM SNP generated 5 - 42 pmol  $\text{NO min}^{-1}$ . The correlation coefficient of the linear fit was  $R^2 = 0.99$ .

It has long been known that NO-emission from SNP is stimulated by light. The mechanism for that photolytical release of NO from SNP has only recently been elucidated (Zhelyaskov and Godwin, 1998). As shown in Figure 13B, in dark, injection of a 4 mM SNP solution into the cuvette did not lead to any measurable generation of NO; obviously the rate of spontaneous SNP decomposition was very low. Illumination resulted in a rapid rise of the NO-emission to reach a steady state (Figure 13B). By addition of a commercial NO-scavenger (c-PTIO; 1 mM), the NO emission was completely abolished in less than 5 min.

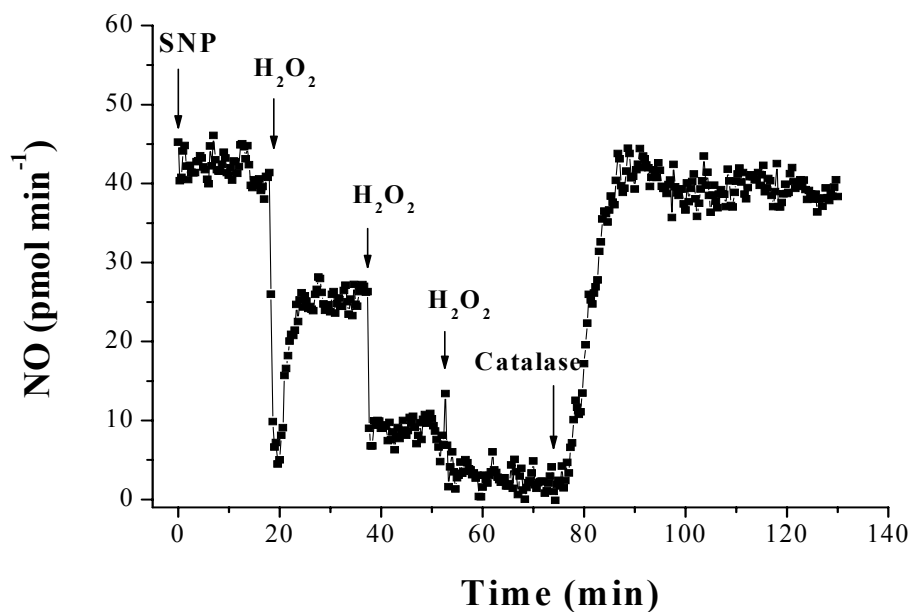


**Figure 13:** Generation of NO by SNP, as quantified by chemiluminescence.

A- Steady-state rates of NO release from solutions containing different SNP concentrations (1 mL buffer solution (50 mM HEPES-KOH, pH 6.5)). The data are means ( $n = 3$ )  $\pm$  SD. B- Time course of NO release from SNP (4 mM) under dark-light condition. NO emission could be completely scavenged by c-PTIO (1 mM), added as indicated. The graph shows one representative experiment.

### 1.2.2 Effect of ROS on NO-emission from an SNP solution

NO might function as an antioxidant directly scavenging ROS that are generated *e.g.* by biological systems. Here, the effects of ROS on NO released from a NO-donor was examined in a simple system. Figure 14 gives the time course of NO release from SNP, as above. As ROS,  $\text{H}_2\text{O}_2$  was added to the buffer solution after NO release induced by 10 mM SNP ( $42 \text{ pmol min}^{-1}$ ) had reached a steady rate. In this representative experiment, NO decreased by approximately 40 % after  $\text{H}_2\text{O}_2$  addition (100  $\mu\text{M}$ ). Repetitive addition of  $\text{H}_2\text{O}_2$  to higher final concentrations finally prevented the NO emission completely. Addition of catalase (20  $\text{U mL}^{-1}$ ) at the end of experiment totally restored the NO signal. The reaction product of NO or of the nitrosium cation ( $\text{NO}^+$ ) with  $\text{H}_2\text{O}_2$  is probably  $\text{ONOO}^-$  (Stamler *et al.* 1992), which is a potent nitrating agent and highly toxic to cells.

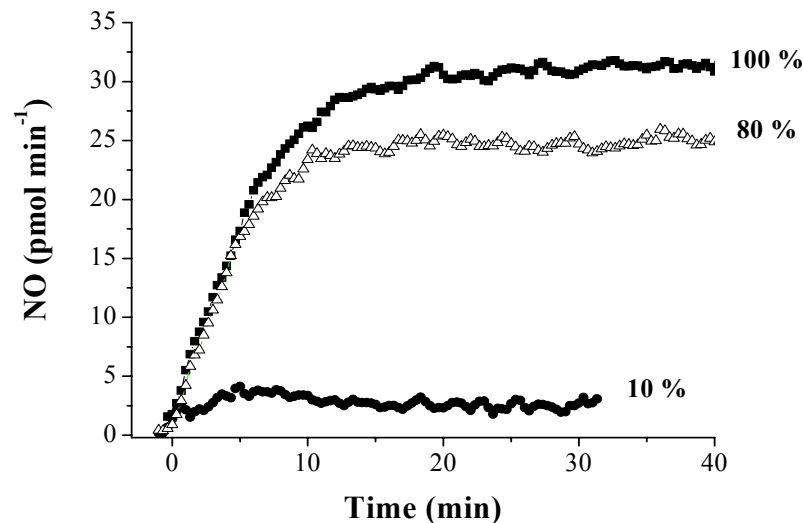


**Figure 14:** Effects of  $\text{H}_2\text{O}_2$  on NO production by a NO-donor (SNP).

Ten mM SNP were added in 1 ml of buffer (50 mM HEPES-KOH, pH 6.5).  $\text{H}_2\text{O}_2$  was repeatedly (three times) injected as indicated to give the following final concentrations: 0.1 mM, 0.5 mM and 1 mM respectively. Catalase ( $20 \text{ U mL}^{-1}$ ) was added at  $T = 75 \text{ min}$ .

### 1.2.3 NO-emission from SNP in cell suspensions

SNP was also used to estimate quenching by cell suspensions of NO under continuous production. As already shown, upon injection of SNP (5 mM) into a simple buffer solution (1 mL), a steady NO emission took place with a rate of  $31 \text{ pmol NO min}^{-1}$  (Figure 15). Injection of SNP into a cell medium (1 mL) without cells indicated a quenching of NO (20 %), but injection into a cell suspension (1 mL) again resulted in strong quenching of NO by cells (compare Figure 11). At steady state NO production, quenching by medium plus cells was around 90 %.



**Figure 15:** Generation of NO by SNP and quenching by cell suspensions.

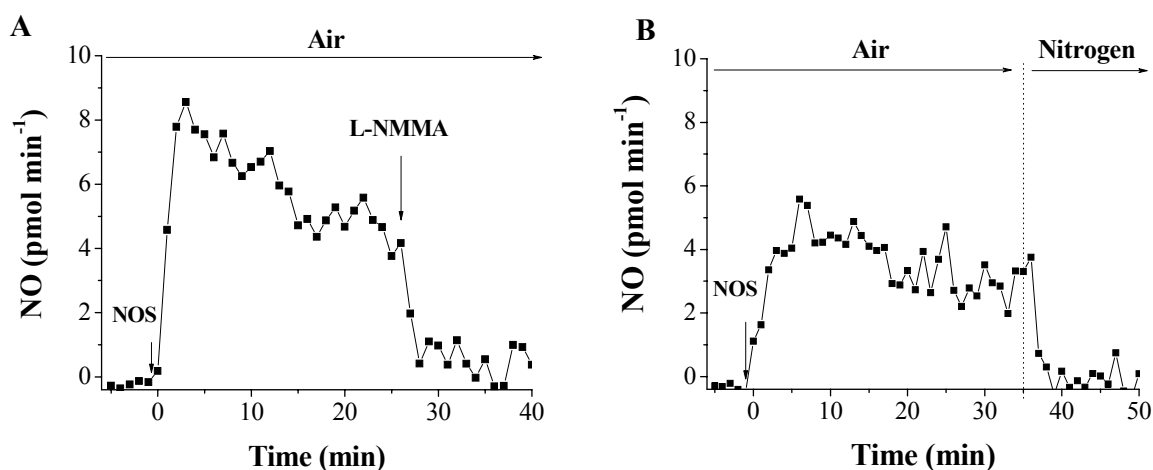
Five mM SNP was injected in 1 mL buffer (10 mM MES, pH 5.5) (■), in 1 mL cells (●) or in 1 mL cell medium (△) into a small cuvette mounted on a shaker. At time  $T = 0$ , SNP was injected. Results are means of five measurements.

## 1.3 NO release from purified enzymes

### 1.3.1 NO-emission from neuronal rat nitric oxide synthase (NOS)

Available literature data for direct quantifying NO formation from purified NOS have been obtained by EPR spectroscopy (Xia and Zweier, 1997; Xia *et al.*, 1998). However, NOS activity has been mostly measured by following the conversion of labelled L-arginine into L-citrulline, and separation of the two compounds by ion exchange. We have directly measured NO production by commercial neuronal rat NOS in our gas-phase chemiluminescence system in order to see how the manufacturer-given NOS activity would correlate with *de facto* NO production (or, whether chemiluminescence would be suited to quantify NOS activity). The experiment was also used to test the effect of commercial NOS-inhibitors on NO production, as these inhibitors will be used in our below-described experiments on the induction of the HR (see chapter 3).

In the absence of the enzyme, no NO was emitted from a solution containing the NOS substrate L-arginine and all cofactors (Figure 16A). After addition NOS (3 U according to the manufacturers instructions), the NO concentration in the headspace increased to a maximum level, resulting in a steady rate of 8 pmol NO min<sup>-1</sup>, which is only 2.7/1000 of the activity given by the manufacturer, probably because of heavy NO scavenging through constituents of the buffer used. NO emission was totally blocked by adding L-NMMA (N<sup>G</sup>-Monomethyl-L-arginine, Monoacetate; 2 mM), which represents a rather high inhibitor concentration (but see our experiments with leaves, Chapter 3). As the NOS-reaction requires oxygen, flushing the cuvette with nitrogen instead of air also completely abolished NO emission (Figure 16B). This is also important, as in many circumstances (described below), we have measured highest NO emission from leaves or cell suspensions under anoxia. The above finding thus excludes any contribution to NO emission of a NOS-like activity, at least under these specific conditions.



**Figure 16:** NO generation from neuronal rat NOS.

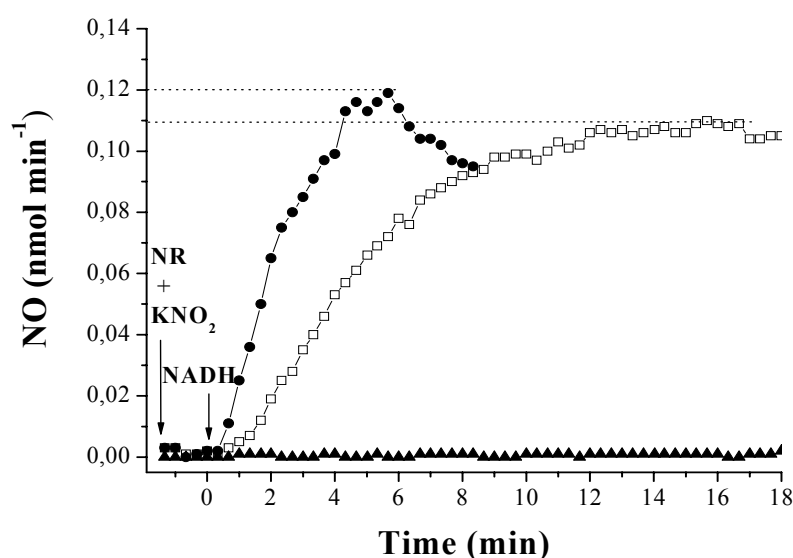
A) The reaction system in air consisted of L-Arg (1 mM), NADPH (1 mM), CaCl<sub>2</sub> (1 mM), calmodulin (1 μM), BH<sub>4</sub> (8 μM), EDTA (0.5 mM), FAD (10 μM), FMN (10 μM), DTT (0.5 mM) in 100 mM HEPES buffer (pH 7.6) with purified neuronal NOS (6 μL containing 3 U = 3 nmol min<sup>-1</sup> according to the manufacturer). After 25 min, the NOS inhibitor (L-NMMA; 2 mM) was added to the buffer. B) Comparison of NOS-activity in air or nitrogen. The reaction was started at T = 0 by adding NOS to the solution containing already cofactors. A rate of NO emission of 10 pmol min<sup>-1</sup> corresponds to 0.15 ppb NO in the gas stream.



### 1.3.2 NO-emission from a solution of purified NR

#### 1.3.2.1 NR-dependent NO production *in vitro*

NR has been shown previously to immediately produce NO when supplied with nitrite and NADH (Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000; Rockel *et al.*, 2002). Indeed, in a stirred buffer solution containing highly purified NR from maize (10 mU) and NADH (200  $\mu$ M), NO emission from the aerated enzyme solution started immediately after nitrite (200  $\mu$ M) addition, and increased continuously to reach a steady state, where the emission rate was 0.11 nmole min<sup>-1</sup> (Figure 17). NRA contained in the solution was determined as 13.8 nmole min<sup>-1</sup> (measured in the presence of excess EDTA). Thus, the rate of NO emission in air was only about 0.80 % of the NRA in air, consistent with previous results (Rockel *et al.*, 2002). In the absence of NR, NO production was not detectable (Figure 17), confirming that chemical NO formation from nitrite was negligible in a physiological buffer at pH 7.6. In nitrogen, steady state NO-emission was slightly higher representing around 0.90 % of NRA. This small difference in NO emission under air and nitrogen could be explained by a reaction of NO with ROS produced by NR (compare Figure 6 and Yamasaki and Sakihama, 2000).



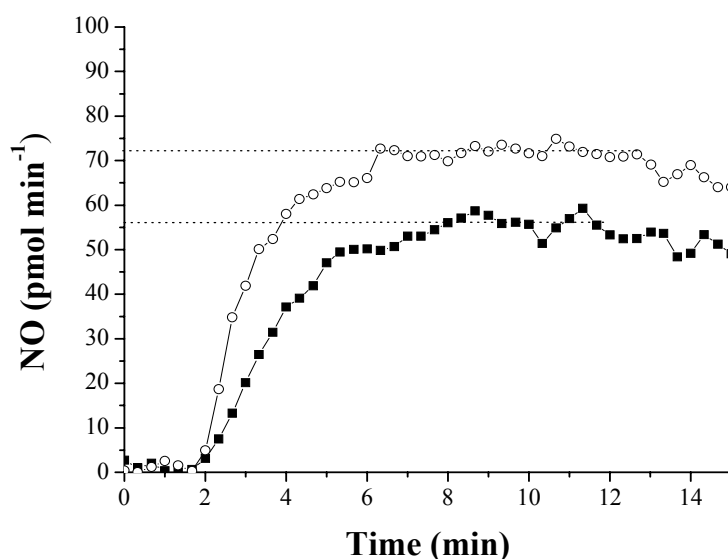
**Figure 17:** NR-dependent NO-emission *in vitro*.

At  $T = 0$ , 200  $\mu$ M NADH were added to 1 ml reaction buffer (100 mM HEPES-KOH, pH 7.6) containing 50 mU of commercially available purified maize NR (Sigma) and 200  $\mu$ M nitrite in air ( $\square$ ), under nitrogen ( $\bullet$ ) and without NR ( $\blacktriangle$ ) in air. Note that this buffer system is more simple (and less scavenging) than in the NOS-experiment (Figure 16).

### 1.3.2.2 Interaction between NO from purified NR and ROS

As mentioned previously, NR can utilize molecular oxygen as an electron acceptor and the product,  $O_2^{\cdot-}$ , is primarily generated *via* the Mo-pterin center (Ruoff and Lillo, 1990; Barber and Kay, 1996). In addition, Yamasaki and Sakihama (2000) showed that NO, produced by purified NR in presence of NAD(P)H, rapidly reacts with  $O_2^{\cdot-}$  to produce the extremely toxic ONOO<sup>-</sup> under aerobic conditions.

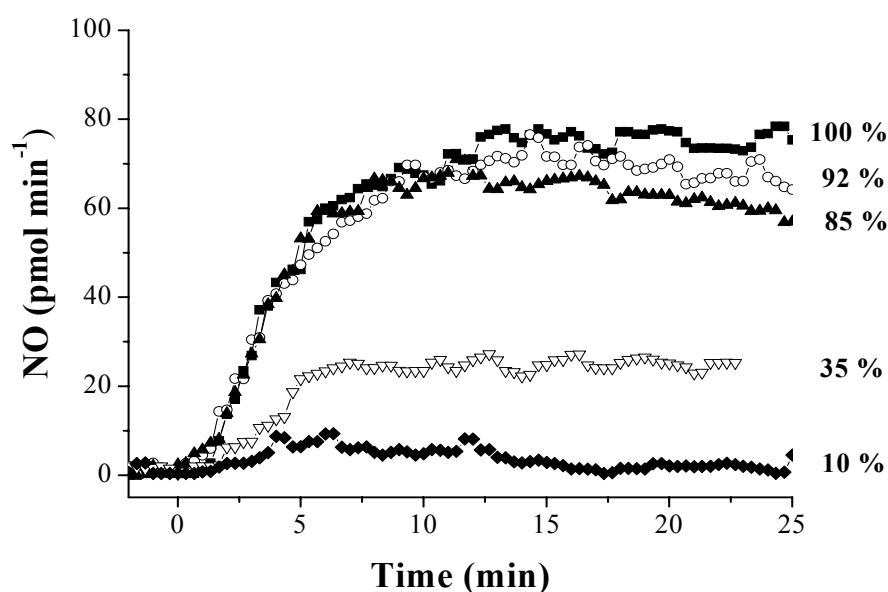
The possibility that  $O_2^{\cdot-}$  produced enzymatically by NR may interfere chemically with NO under aerobic conditions and thus impair NO detection by chemiluminescence, was examined by adding antioxidant enzymes to the solution in order to scavenge ROS. Steady-state NO-emission from NR in presence of SOD and catalase ( $20\text{ U mL}^{-1}$  each) was  $72\text{ pmol min}^{-1}$ , whereas without these enzymes it was  $56\text{ pmol min}^{-1}$  (Figure 18). Thus, more than 20 % of the NO produced by NR was trapped by ROS also derived from NR. The normal NRA of the purified NR enzyme was not affected by the presence or absence of the antioxidant enzymes. In the particular case, this activity was  $7.5\text{ nmol min}^{-1}$ , showing that the NO-emission represented 0.95 % and 0.75 % of the NRA with and without antioxidant enzymes, respectively.



**Figure 18:** Effect of antioxidant enzymes on NO-emission from a NR-purified enzyme.

At  $T = 2\text{ min}$ ,  $200\text{ }\mu\text{M}$  NADH were added to  $1\text{ ml}$  reaction buffer ( $100\text{ mM}$  HEPES-KOH pH 7.6) containing  $25\text{ mU}$  of commercially available purified maize NR (Sigma) and  $200\text{ }\mu\text{M}$  nitrite (■), plus catalase and SOD ( $20\text{ U mL}^{-1}$  each) (○). Data are means ( $n = 3$ )  $\pm$  SD.

While the above data do not allow a differentiation between effect of  $\text{H}_2\text{O}_2$  or  $\text{O}_2^-$  on apparent NO production by NR, we have also directly examined how  $\text{H}_2\text{O}_2$  would affect NO emission (compare Figure 14). This is the more important, as both, NO and  $\text{H}_2\text{O}_2$  can cross cell membranes to serve not only as precursors of more reactive species in adjacent cells, but also as intermediary signals. *In vitro* studies have suggested that a reaction between gaseous NO and  $\text{H}_2\text{O}_2$  produces singlet oxygen or hydroxyl radicals, a highly cytotoxic species (Noronha-Dutra *et al.*, 1993; Nappi and Wass, 1998). The potential trapping of NO by  $\text{H}_2\text{O}_2$  was checked in solution containing only buffer (100 mM HEPES-KOH, pH 7.6), purified NR (10 mU) and substrates (nitrite and NADH). This system was co-injected with different concentrations of  $\text{H}_2\text{O}_2$ , and NO-emission was followed (Figure 19). NO emission from NR solution was weakly affected by 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (8 % of the NO-emission lost) and by 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (15 % of NO-emission lost). However, at higher concentrations (100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 250  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ), NO emission was strongly quenched. These results add to our previous suggestion  $\text{H}_2\text{O}_2$  could add to an underestimation of NO production if it would exist inside cells within the above concentration range (up to 100  $\mu\text{M}$ , see Veljovic-Jovanovic *et al.*, 2002).



**Figure 19:** NR-dependent NO-emission *in vitro* is quenched by  $\text{H}_2\text{O}_2$ .

At zero time, 200  $\mu\text{M}$  (final concentration) nitrite was added to 1 ml reaction buffer (100 mM HEPES-KOH pH 7.6) containing 10 mU of commercially available purified maize NR (NECi) and 200  $\mu\text{M}$  NADH and various concentrations of  $\text{H}_2\text{O}_2$  in air: control (■); 10  $\mu\text{M}$  (○); 50  $\mu\text{M}$  (▲); 100  $\mu\text{M}$  (▽) and 250  $\mu\text{M}$  (◆).

Here again, H<sub>2</sub>O<sub>2</sub> addition did not affect normal NRA (Table I). Thus, the differences in the NO emission rates were not due to direct inhibition by ROS of NR, but to trapping of NO.

**Table I:** Effects of H<sub>2</sub>O<sub>2</sub> on NRA

The activity of purified NR was determined in EDTA-buffer with different concentrations of H<sub>2</sub>O<sub>2</sub>.

	NRA (nmol min <sup>-1</sup> )
NR	8.47
NR + 20 μM H <sub>2</sub> O <sub>2</sub>	8.17
NR + 100 μM H <sub>2</sub> O <sub>2</sub>	7.83

## 1.4 Summarizing conclusions

According to the above data, chemiluminescence detection seems to be a suitable method to detect NO production from solutions of NO donors or NO producing enzymes with high specificity and sensitivity. The data have also indicated that considerable quenching of NO production may occur, as for example in cell suspensions. To a small part, this NO scavenging may be an oxidative one, eventually involving ROS.

*In vitro*, NO could be formed enzymatically from purified NR, confirming previous results (Yamasaki *et al.*, 1999; Rockel *et al.*, 2002). Importantly, and contrary to certain literature suggestions (Dean and Harper 1988), even at saturating substrate concentrations (nitrite and NADH), NO production appeared to be only a small fraction (around 1 %) of the nitrate reducing capacity of NR *in vitro*.

## CHAPTER 2

### **NO production by plants cells, under non-stress conditions**

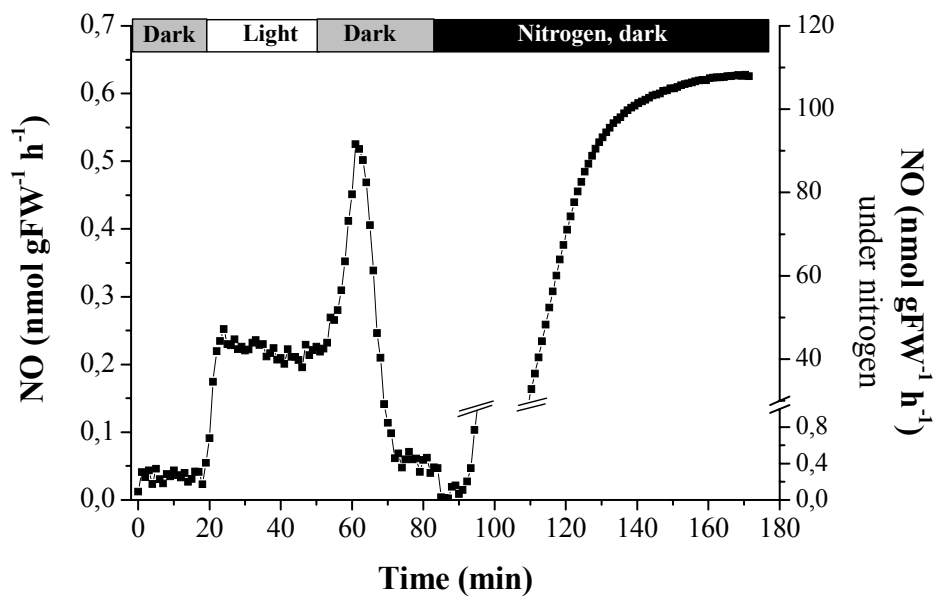
As already pointed out in the introduction, plants, in contrast to animals, have probably several systems to produce NO. It is, however, still controversial which of these systems is active under specific situations, and how much one or the other system actually contributes to NO production. It has been shown by several groups that the cNR or iNR of plants reduce nitrite to NO in a side-reaction, using NADH as electron donor (Dean and Harper, 1988; Yamasaki *et al.*, 1999; Yamasaki and Sakahima, 2000; Rockel *et al.*, 2002). The  $K_m$  for nitrite has been shown to be 100  $\mu\text{M}$ , and the reaction was competitively inhibited by nitrate ( $K_i = 50 \mu\text{M}$ ) (Rockel *et al.*, 2002). All these data have established the opinion that NR is an important and sharply regulated NO source in plants. However, Stöhr *et al.* (2001) has shown a plasma membrane (PM)-associated NO formation, depending on nitrite but not on NADH. This NO formation activity was not caused by a PM-NR but by a hitherto unknown enzyme, which was termed Nitrite: NO oxido-reductase (NI-NOR). In addition, XO/XDH has been suggested to reduce nitrite to NO under anoxic conditions in the presence of NADH (Millar *et al.*, 1998; Godber *et al.*, 2000b; Li *et al.*, 2001). The involvement of a NOS-like activity was much more debatable, as much of the available evidence was based on pharmacological studies with NOS inhibitors, or on immunological evidence obtained with antibodies of questionable specificity (Lo *et al.*, 2000; Butt *et al.*, 2003). On the other hand, a most recent paper by Chandok *et al.* (2003) has given the first direct and unequivocal evidence that at least after challenge by TMV, tobacco leaves would slowly induce an iNOS, which was identified as part of the leaf mitochondrial GDC complex. Still, it is an unsolved problem how much the different NO producing systems contribute to NO signalling in one or the other situation, and whether or not these systems interfere with each other. In the following chapter, we will focus attention first on NR as the major NO source in “normal”, non stressed plants. The modulation of NR-dependent

NO production by various external and internal (“limiting”) factors will be examined. For this purpose, NR and NiR expression was modified in tobacco plants and cell suspension cultures grown on nitrate and ammonium as N-source, and NR and NiR deficient mutant plants were also used to demonstrate the relation between NR, NiR and NO.

## 2.1 NO-emission from nitrate grown wild-type plants and cell suspensions

### 2.1.1 Regulation of NO-emission in vivo

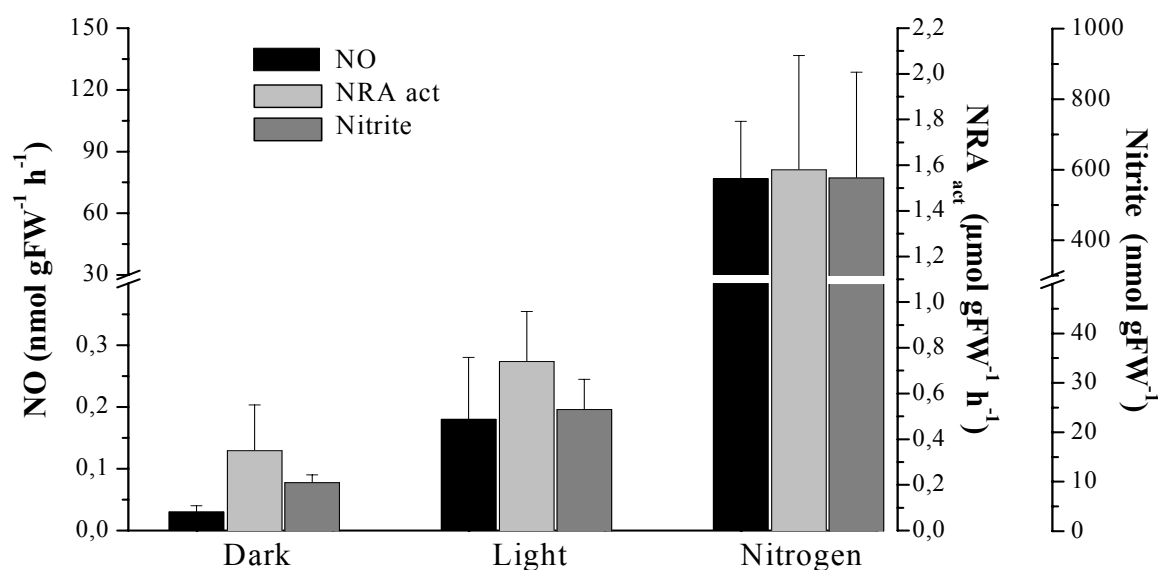
NO production from detached tobacco leaves (*Nicotiana tabacum* var. *Xanthi*, if not mentioned otherwise) was continuously followed as NO emission into the gas phase by chemiluminescence detection. Figure 20 shows a typical time-course of a dark-light-dark pattern from tobacco leaves. During darkness in air, detached tobacco leaves, with the petioles in nitrate solution (10 mM), emitted only very low amounts of NO, but in the light, NO production increased about five fold to a constant rate of  $0.25 \text{ nmol gFW}^{-1} \text{ h}^{-1}$ . When the light was suddenly switched off, NO release increased transiently but again settled to a very low steady-state value in the dark. This “light-off burst” of NO emission was routinely observed not only with tobacco leaves, but also with other species like spinach or *Vicia faba* (data not shown). Much higher NO-emission occurred when darkened leaves were kept under anoxia in an atmosphere of nitrogen. Under these conditions, NO emission was  $\sim 100 \text{ nmol gFW}^{-1} \text{ h}^{-1}$ , which is more than 1000 times higher than in air (dark).



**Figure 20:** NO-emission pattern of non-elicited detached tobacco leaves during a normoxic-anoxic transient. NO-emission ( $\text{nmol gFW}^{-1} \text{h}^{-1}$ ) was measured in the chamber flushed with air (dark or light) or with nitrogen in dark. Data show one experiment, representative of six replicates.

The relation between NO-production and NRA was more closely investigated. Figure 21 compares NRA, nitrite contents and NO emission in tobacco leaves in the above (Figure 20) transient situations. Nitrite concentrations were very low in the dark ( $9 \text{ nmol gFW}^{-1}$ ), but increased in the light ( $25 \text{ nmol gFW}^{-1}$ ). Usually, in the dark, NR is less active than in the light, and photosynthetic electron transport to NiR is absent (no nitrite is reduced). In light, NR is activated and the nitrite concentration increases to a new steady state (Huber *et al.*, 1992; Kaiser *et al.*, 1992), although nitrite is now reduced by photosynthetic electron transport. NRA assayed in the presence of  $\text{Mg}^{2+}$  ( $\text{NRA}_{\text{act}}$ ), was about  $0.35 \text{ } \mu\text{mol gFW}^{-1} \text{h}^{-1}$  and increased to  $0.74 \text{ } \mu\text{mol gFW}^{-1} \text{h}^{-1}$  in the light. “The light-off peak” (Figure 20) reflects a transient nitrite accumulation ( $34 \text{ nmol gFW}^{-1}$ ) (compare Riens and Heldt, 1992). As a possible explanation, it has been suggested that upon a sudden light off, nitrite reduction stops immediately whereas nitrate reduction continues for a while since NR inactivation is slow (Kaiser *et al.*, 1993).

Under anoxia in the dark, the very high NO-emission was correlated with an activation of NR, reaching 4.5 and 2 times the activity in the dark (air) and in light (air) respectively. More important, nitrite was heavily accumulated, reaching (in the above example) 480 nmol gFW<sup>-1</sup> (50 fold more than in air, dark). Under anoxia, nitrite reduction stops (compare Botrel and Kaiser, 1997), but at the same time NR is hyper-activated and NADH supply for NR is probably at maximum (for further information see Discussion). By and large, NO release *in vivo* followed the pattern of NRA, but it appeared to be quantitatively more related to nitrite accumulation in the tissue. The maximum rate of NO release was only a small fraction (0.025 % in light, air) of the NRA in the extract measured in presence of magnesium. This result was consistent with previous reports, where NO-emission from spinach leaves represented a small fraction of the total NRA in the tissue (about 0.01 %-0.1 %) (Rockel *et al.*, 2002). Under nitrogen, where both NADH and nitrite are assumed to reach saturating concentrations, this ratio increased to 4.8 %. Unexpectedly, NO production as percent of the NR capacity under anoxia was higher than the 1 % obtained with purified maize NR *in vitro*, which was also substrate saturated.



**Figure 21:** Response of NO-emission, NRA<sub>act</sub> and nitrite content of tobacco leaves grown with nitrate nutrient solution to a normoxic-anoxic transition.

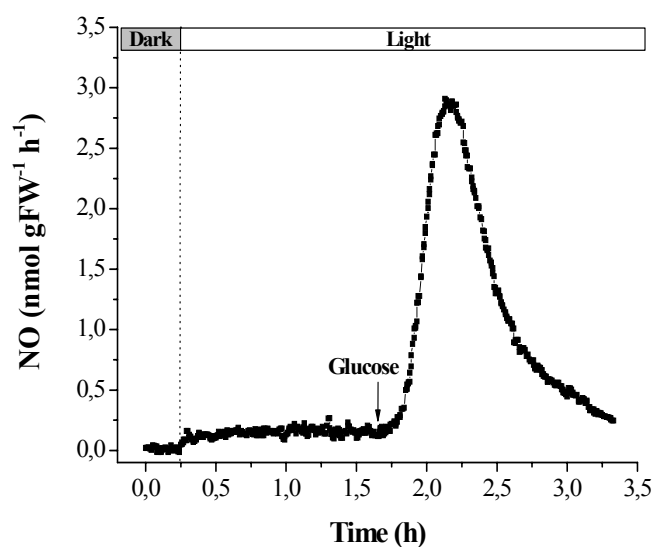
Samples (leaf discs) for determination of NRA and nitrite content were taken after 30 min in the dark, 60 min in light or 60 min under nitrogen from separate leaves, kept under identical conditions as for the NO measurement (n = 4, ± SD).



It has been shown that NR could be artificially activated by the absence of oxygen, *e.g.* under sudden anoxia in roots, NR was activated within minutes (Botrel *et al.*, 1996; Botrel and Kaiser, 1997). This activation was due to the dephosphorylation of NR (Glaab and Kaiser, 1993; Kaiser and Brendle-Behnisch, 1995). NRA was also modulated by several other external factors such as sucrose feeding, uncoupler treatment or AICAR feeding (see next page), all leading to an activation of NR (Glaab and Kaiser, 1995; Kaiser and Brendle-Behnisch, 1995). Therefore, it was examined whether these other treatments would also lead to an additional NO production.

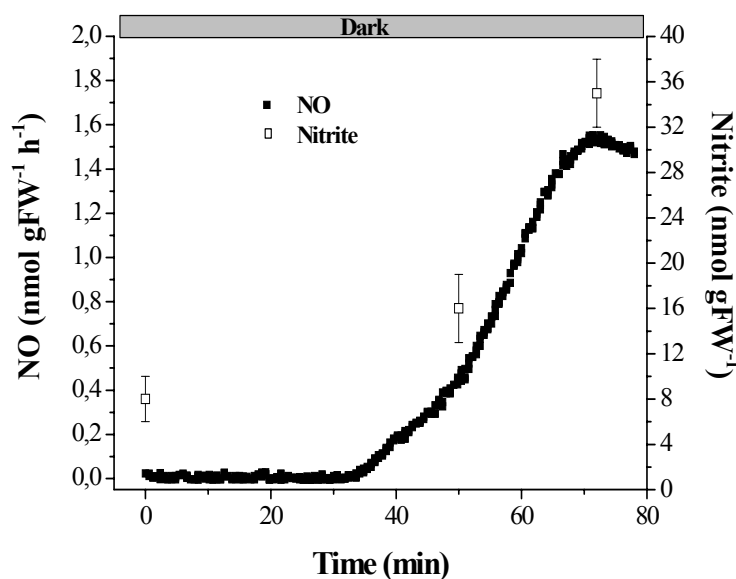
NR is regulated transcriptionally and post-translationally in response to signals from nitrogen and carbon metabolism. Both, carbohydrates produced directly by photosynthetic fixation of CO<sub>2</sub> or from the breakdown of stored carbon may activate NR (Kaiser and Brendle-Behnisch, 1991). Glucose feeding stimulated NRA and increased nitrite accumulation, followed by boosting respiration. Degradation of the NR protein in darkness was also prevented by glucose feeding (Cheng *et al.*, 1992; de Cires *et al.*, 1993; Lillo, 1994, Morcuende *et al.*, 1998).

In Figure 22, detached tobacco leaves with the petiole in water were fed with an external carbon source (glucose) through that solution during the light period to promote transpiration and xylem transport. The addition of 2 mM glucose brought about a transient NO emission reaching a level of 3 nmol gFW<sup>-1</sup> h<sup>-1</sup> after 30 min and declined slowly thereafter.



**Figure 22:** Effect of glucose on NO emission by tobacco detached leaves. Leaves were first kept with the petiole in water. Where indicated, 2 mM glucose was added. Data show one experiment out of two repetitions with almost identical results.

The dephosphorylation/activation of phospho-NR, catalyzed by endogenous protein phosphatases, can be stimulated *in vitro* by 5'-AMP (Kaiser and Spill, 1991; Kaiser *et al.*, 1992). AICAR (5-Aminoimidazole-4-carboxamide 1- $\beta$ -D-ribose nucleoside) has been used in animal physiology as a tool to mimic effects of 5'-AMP *in vivo*. AICAR is cell permeable and becomes phosphorylated to ZMP (5-Aminoimidazole-4-carboxamide 1- $\beta$ -D-ribose nucleoside 5'-monophosphate), an analog of 5'-AMP (Corton *et al.*, 1995). It has been shown in plants that AICAR activates NADH-NR *in vivo*, concomitantly with the accumulation of ZMP in the tissue (Huber and Kaiser, 1996; Kaiser and Huber, 1997) and slows down NR degradation (Kaiser and Huber, 1997). Although its mode of action is not precisely known, AICAR appears to interact directly with binding of 14-3-3 proteins to phospho-NR and thereby releases the inhibition of NR (Athwal *et al.*, 2000). These results with NR led us to ask whether AICAR feeding could also increase NO-emission *via* NR activation. Tobacco leaves were excised from plants early in the photoperiod, the cut petioles were put into water and the leaves were placed in the dark at 25°C. After 40 min, to allow complete inactivation of NR by phosphorylation (Huber *et al.*, 1992), detached leaves were placed in a solution containing 10 mM AICAR, and NO production was monitored in dark. As shown in the Figure 24, an increase in NO emission was observed and reached a steady state at 1.6 nmol gFW<sup>-1</sup> h<sup>-1</sup> after more than 1 h of AICAR feeding. The nitrite content increased concomitantly with the NO production (Figure 23).



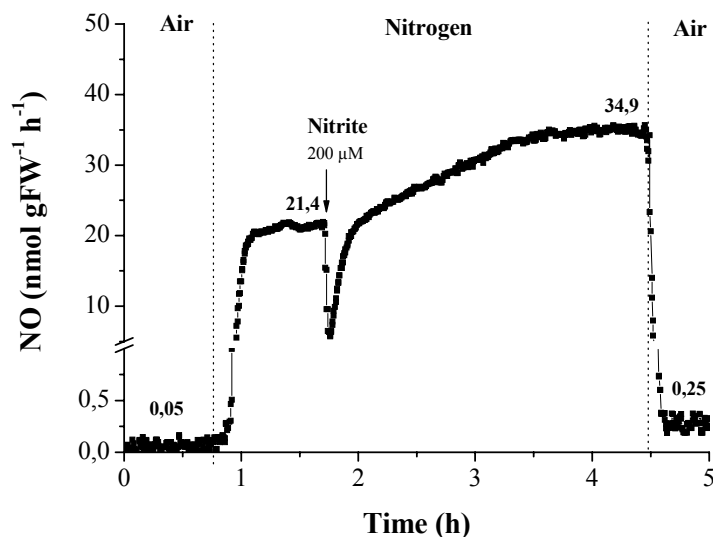
**Figure 23:** NO-production *in vivo* and nitrite content after AICAR feeding.

After a period of darkness to inactivate NR, detached tobacco leaves were fed with 10 mM AICAR at T = 0. Samples for nitrite determination were leaf discs (10 mm diameter) punched out from the same leaves (n = 3,  $\pm$  SD).

### 2.1.2 NO-emission from cell suspensions: evidence that cytosolic NADH limits NO emission

Suspension cultures are morphologically more homogeneous than leaves or roots and thus, allowing a more uniform response to any change of growth conditions or treatments. Further, addition of chemicals is rapid, uniform and precise and the same holds for taking aliquots from the suspension for various analytical purposes. These practical advantages more than balance the increased input of labour required to maintain the cultures.

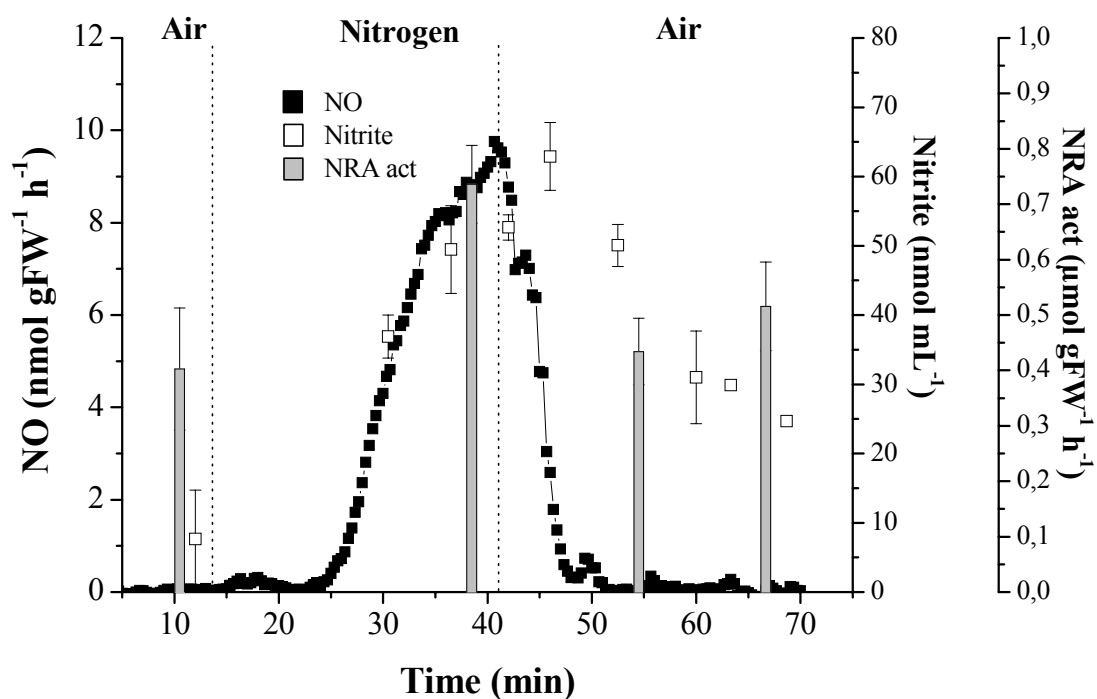
Using non-green nitrate-grown tobacco cell suspensions, NO-emission was measured following a typical air-nitrogen transient. Ten mL of the cells in a Petri dish were mounted in a transparent cuvette on a shaker. NO-emission in air during continuous gentle shaking was very low ( $0.05 \text{ nmol NO gFW}^{-1} \text{ h}^{-1}$ ) (Figure 24). Under nitrogen, as in darkened leaves, NO emission increased rapidly, reaching a steady state after a few minutes ( $21.4 \text{ nmol NO gFW}^{-1} \text{ h}^{-1}$ ). Addition of nitrite to the suspension increased the rate of NO emission ( $34 \text{ nmol NO gFW}^{-1} \text{ h}^{-1}$ ), indicating that it was not yet nitrite saturated. After return to aerobic conditions, the NO-emission rate (in the presence of nitrite) dropped to a somewhat higher steady state than before nitrite addition ( $0.25 \text{ nmol NO gFW}^{-1} \text{ h}^{-1}$ ). Thus, even with nitrite added, aerobic NO emission from cell suspensions was quite low.



**Figure 24:** NO-emission of nitrate-grown tobacco cell suspensions.

Cell suspensions were flushed by air or nitrogen gas stream.  $\text{KNO}_2$  ( $200 \mu\text{M}$ ) was added to the cell culture as indicated. A representative graph out of three identical experiments with similar results.

In order to understand the background behind the strong decline of nitrite-dependent NO production in air, NRA (+Mg<sup>2+</sup>) and nitrite concentrations (medium + cells) were determined during a nitrogen/air transition (Figure 25). It is obvious that the drastic drop of NO-emission after switching from nitrogen to air, was not correlated with the nitrite content, which remained still high after 30 min in air (around 25 nmol NO<sub>2</sub> gFW<sup>-1</sup>). The NRA also did not change much after 30 min, indicating that some other factor was strongly limiting NO formation in air. As in leaves, NO-emission was about 1.5 % of NRA when cells were anoxic and had accumulated nitrite.



**Figure 25:** NO-emission, NRA<sub>act</sub> and nitrite content of cell suspensions during an air-nitrogen transient.

At the time indicated, aliquots (0.5 mL) were removed from the suspensions for NRA and nitrite determination. Data for NRA and nitrite are means ( $n = 3 \pm \text{SD}$ ). The NO-emission curve is a representative one out of three similar curves.

## 2.2 NO-emission from NR-free leaves and cell suspensions

NRA has been demonstrated to be nitrate-inducible in higher plants (Daniel-Vedèle and Caboche, 1996; Sivasankar and Oaks, 1996). Accordingly, growth of plants in the complete absence of nitrate, but on ammonium as N-source can be used to produce plants which are virtually free of NR, and also of NiR. Such plants may still contain other MoCo enzymes, *e.g.* xanthine dehydrogenase and/or aldehyde oxidase, which are also potential candidates to produce NO from nitrite. Like NR, those enzymes are still expressed but non-functional when plants are grown with tungstate instead of molybdenum. “Tungstate plants” are free of any MoCo-enzyme activities and thus permit an investigation the involvement of MoCo-enzymes in NO production. Tobacco has two *nia* genes (Müller, 1983). A completely NR-deficient double mutant (*nia30*) provides another useful tool to study the involvement of NR in NO emission.

Thus, in these experiments, we used the following plant systems:

- a) Ammonium-grown plants without NR and NiR;
- b) Nitrate plus tungstate-grown plants without any MoCo-enzyme, but with NiR;
- c) Ammonium plus tungstate-grown plants without any MoCo-enzyme and without NiR;
- d) *nia 30* double mutants without NR, but with all others.

### 2.2.1 Ammonium-grown plants

With leaves from tobacco plants cultivated in hydroponics on ammonium in the complete absence of nitrate, NRA (soluble) was extremely low and hardly detectable, and the nitrite content was also below detection, even under anoxia (Table II). As the accumulation of nitrite appeared an indispensable prerequisite for NO production, it was therefore expected that such plants would not emit NO (Table II), which was indeed the case.

**Table II:** NRA, nitrite content and NO-emission of ammonium-grown tobacco leaves.

Samples (leaf discs) for NRA<sub>act</sub> ( $\mu\text{mol gFW}^{-1} \text{h}^{-1}$ ) and nitrite ( $\text{nmol gFW}^{-1}$ ) determination were taken after 30 min in the dark, 60 min in light or 60 min under nitrogen from separate leaves but kept under the same conditions as for the NO measurements ( $\text{NO}$ ;  $\text{nmol gFW}^{-1} \text{h}^{-1}$ ) ( $n = 3, \pm \text{SD}$ ) (N.D. = not detected).

	<b>NRA<sub>act</sub></b>	<b>Nitrite</b>	<b>NO</b>
<b>Dark</b>	0.012 $\pm$ 0.01	N.D.	N.D.
<b>Light</b>	0.016 $\pm$ 0.01	N.D.	N.D.
<b>Nitrogen</b>	0.010 $\pm$ 0.01	N.D.	N.D.

It should be mentioned that leaves from tobacco grown on ammonium (3 mM) plus nitrate (10 mM) had similar nitrite contents, NRA and emitted NO just like tobacco grown on nitrate only (data not shown). Thus, there was no direct interference of ammonium cultivation with NO emission.

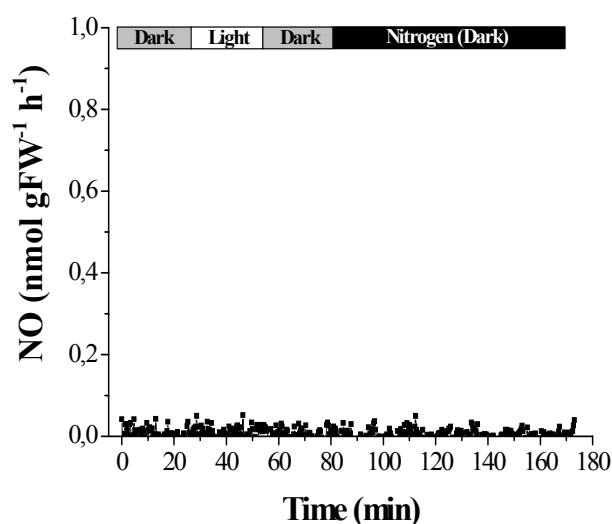
### 2.2.2 *Plants grown on nitrate plus tungstate*

As has been pointed out above, replacement of molybdate by tungstate (500  $\mu\text{M}$ ) in a nitrate-containing nutrient solution should result in plants with non-functional NR, XDH and AOX (alternative oxidase), while NiR activities should be normal. After five days on tungstate, NRA<sub>act</sub> in illuminated tobacco leaves (*N. tabacum* cv. *Xanthi*) became very low (Table III), representing only 1.6 % of the control. Activities of other MoCo enzymes were not measured.

**Table III:** Effect on tungstate on NRA and nitrite content in tobacco leaves under light conditions. NRA<sub>act</sub> and nitrite content from tungstate treated leaves (during five days) were expressed as a percentage of controls (+ molybdenum). Results are means of three measurements. NRA<sub>act</sub> and nitrite content for the control plants was 0.815  $\mu\text{mol gFW}^{-1} \text{h}^{-1}$  and 26.2  $\text{nmol gFW}^{-1}$ , respectively.

	NRA <sub>act</sub> (%)	Nitrite (%)
<b>Control</b>	100	100
<b>Tungstate</b>	1.6	N.D.

As expected, no NO emission could be detected, neither in the light nor in the dark under anoxia (Figure 26). Clearly, active NR was an absolute requirement for a measurable NO emission and for nitrite accumulation. As mentioned, XO/XDH has been shown to produce NO from nitrite (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000b; Li *et al.*, 2001). However, without exogenous nitrite, NR would be the only source for nitrite. Thus, even if some residual XDH activity would exist in tungstate-fed plants, it could not produce NO without NR.

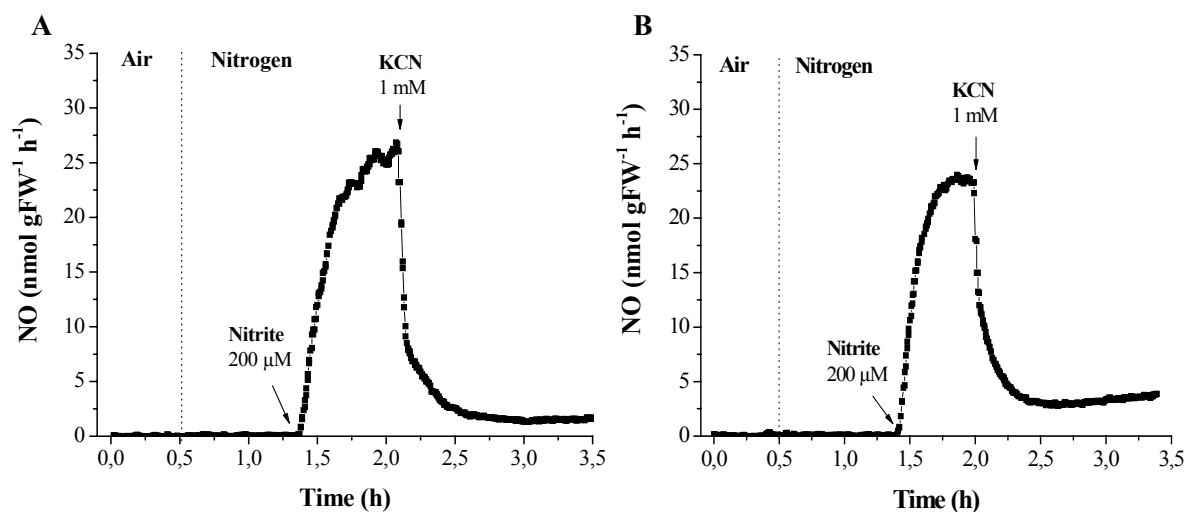


**Figure 26:** Absence of NO-emission from tungstate-grown detached tobacco leaves during a dark-light-dark-N<sub>2</sub> transient.

Others conditions are as in Figure 20. Data show one experiment, representative of five replicates.

### 2.2.3 Ammonium +/- tungstate- grown cell suspensions

Like in leaves, NR-free cells (ammonium cells) did not produce NO in air and under nitrogen (Figure 27A). However, when nitrite (200  $\mu\text{M}$ ) was added under nitrogen, these cells produced NO in a large amounts (26  $\text{nmol NO gFW}^{-1} \text{h}^{-1}$ ), almost similar to nitrate cells under anoxia. This anaerobic NR-independent NO production was almost completely inhibited by 1 mM KCN, a potent but unspecific inhibitor of heme enzymes. The cyanide inhibition can be taken as proof that this NO emission was not due to non-enzymatic NO-production from nitrite, which may occur if somewhere in the cell the pH would drop below pH 5. In order to check whether this nitrite dependent, anoxic NO-emission was produced by a MoCo enzyme, “ammonium cells” were grown in absence of molybdate but in presence of tungstate (100  $\mu\text{M}$ ) in the medium. However, as shown in Figure 27B, replacement of molybdate by tungstate had no effect on nitrite-dependent NO emission. Thus, under anoxia cells were able to produce NO from nitrite in a MoCo-independent, cyanide-sensitive pathway.



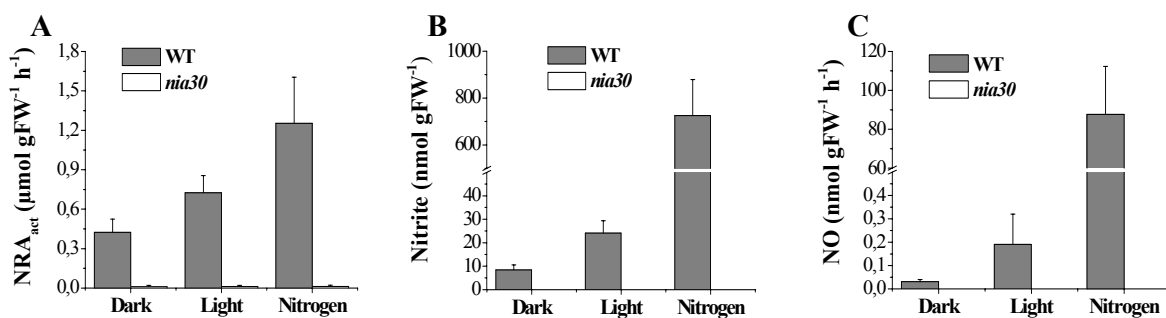
**Figure 27:** NO-emission of ammonium- and tungstate-grown tobacco cell suspensions.

A- Ammonium-grown cell suspensions. B- Ammonium + tungstate -grown cell suspensions. Other conditions as described in Figure 26.  $\text{KNO}_2$  (200  $\mu\text{M}$ ) and KCN (1 mM) were added to the cell suspension as indicated.



### 2.2.4 NR-deficient tobacco mutants

In our experiments, a NR-defective double mutant of tobacco (*nia 30*) was cultivated most of the time on ammonium (hydroponics). Three days before the experiments, plants were transferred to nutrient solutions containing nitrate. The mutant leaves were totally devoid of NRA (Figure 28A) and contained no nitrite (Figure 28B). As expected, NO-emission failed to be detectable (Figure 28C), in contrast with the WT (*N. tabacum* cv. Gatersleben). That confirms the above results that NO-emission was strictly dependent on nitrite, and therefore on NRA.



**Figure 28:** NRA<sub>act</sub>, nitrite content and NO-emission in WT (*N. tabacum* cv. Gatersleben) and *nia30* leaves in dark and light (air) and dark (nitrogen).

White bars are too low to be drawn. Samples (leaf discs) for NRA and nitrite determination were taken after 30 min in dark, 45 min in light and 60 min in nitrogen (n = 3 ± SD).

## 2.3 NO-emission from an antisense nitrite reductase tobacco mutant (clone 271)

Rockel *et al.*, (2002) reported that aerobic NO-emission from sunflower or spinach leaves was generally only a small fraction of the total NRA in the tissue (about 0.01-0.1 %). This is because nitrite is efficiently converted to ammonia by the activity of nitrite reductase (NiR) in the chloroplasts, and does not reach the concentrations required for high NO production. As nitrite is cytotoxic to plants, a complete inhibition of NiR activity is thought to be lethal for the plant cells due to the large accumulation of nitrite (Vaucheret *et al.*, 1992). The tobacco clone 271, expressing a nitrite reductase (NiR) antisense RNA under

the control of the CaMV 35S promoter, was used to more closely examine the relation between NiR and NO production at an unchanged NR expression and activity.

When the transgenic line 271 and WT were grown on nitrate during one week, NR activities were similar in clone 271 and WT (Table IV). However, the nitrite content of clone 271 was much higher than that of the WT, around 5 times more in dark (air) and 10 to 15 times higher under light conditions. Importantly, the nitrite content of WT leaves and of the transformant leaves was very similar under nitrogen. As the mutant leaves accumulated much more nitrite than WT leaves under normal conditions, rates of NO emission were expectedly higher than in WT leaves. According to that, clone 271 already exhibited a high NO emission in the dark, (up to 10-fold higher than in WT). Most important, the NO emission rates and nitrite contents of illuminated transformant leaves were 100 fold higher than in WT, while in the dark under anoxia, WT and clone 271 exhibited rather similar NO emission and nitrite concentrations.

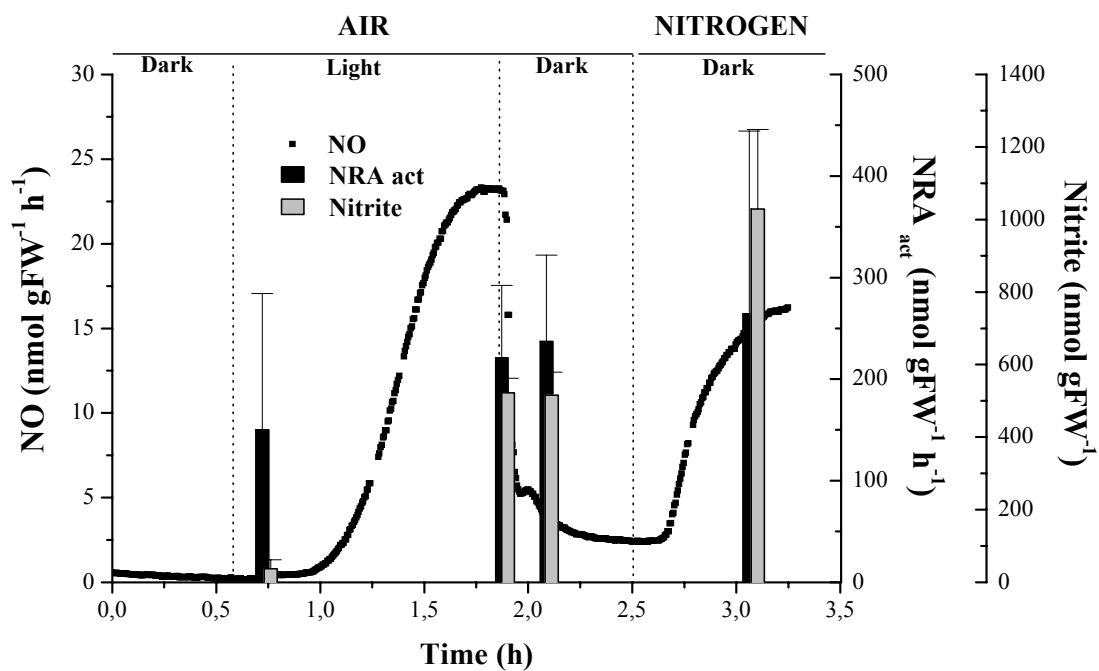
NO-emission from illuminated mutant leaves was around 4.15 % (and occasionally up to 10 %) of the NR capacity. This value is significantly higher than with purified NR *in vitro* (1 %). The reason is unknown, but generally those values indicate that NO scavenging by leaves was probably low (see 2.4).

**Table IV:** NRA<sub>act</sub>, nitrite concentrations and NO emission of NiR deficient detached leaves of clone 271 and of WT (*N. tabacum* cv. Gatersleben) in light, in dark (air) and in dark (N<sub>2</sub>).

NO emission is also given as a percentage of the nitrate reduction capacity. Samples for NRA, nitrite determination and NO were taken after 45 min in the dark, 60 min in light or 60 min under nitrogen (mean of four samples ± SD).

	NRA ( $\mu\text{mol gFW}^{-1} \text{h}^{-1}$ )	Nitrite ( $\text{nmol gFW}^{-1}$ )	NO ( $\text{nmol gFW}^{-1} \text{h}^{-1}$ )	NO/NRA %
<b>WT leaf</b>				
<i>Air (dark)</i>	0.4 ± 0.2	8.6 ± 2.4	0.04 ± 0.01	0.01
<i>Air (light)</i>	0.8 ± 0.2	<b>28.1</b> ± 6.0	<b>0.21</b> ± 0.1	0.03
<i>Nitrogen</i>	1.4 ± 0.5	574 ± 292	74.5 ± 26.1	<b>5.32</b>
<b>Line 271 leaf</b>				
<i>Air (dark)</i>	0.22 ± 0.1	38 ± 28	0.91 ± 0.5	0.41
<i>Air (light)</i>	0.75 ± 0.3	<b>319</b> ± 174	<b>31.10</b> ± 16.2	<b>4.15</b>
<i>Nitrogen</i>	0.86 ± 0.3	634 ± 307	21.49 ± 8.9	2.50

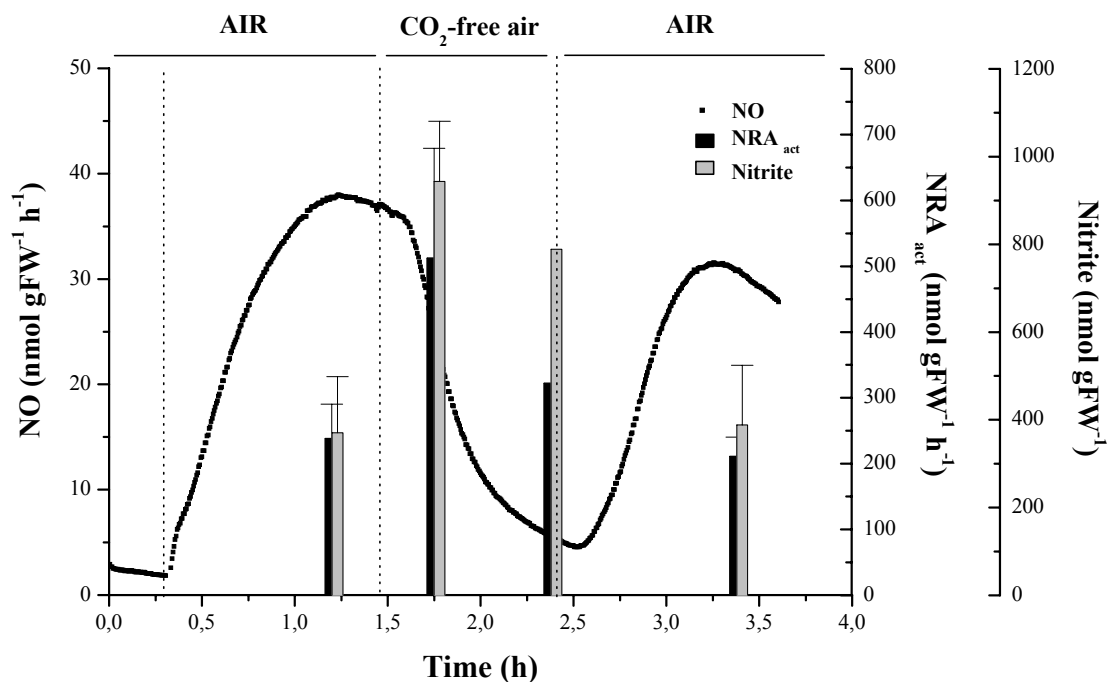
It has been previously suggested that the above increase in the NO emission upon a dark-light transition is mainly a consequence of the light-activation of NR. However, as already pointed out, NO emission is not only depending on nitrate reduction, but also on nitrite reduction, which together determine the tissue nitrite concentration. In clone 271, variations in NO-emission are not due to the changes in nitrite reduction rates, but only to changes in nitrate reduction *in situ*. NO emission of 271 leaves during a dark-light-dark transition (Figure 29) was very high in the light, but broke down almost immediately in a subsequent dark phase. Unexpectedly, NRA (+ Mg<sup>2+</sup>) and nitrite concentrations in those leaves (clone 271) remained high for some time after switching the light off (Figure 29). Thus, the drastic and rapid decrease of NO emission during the light-dark transient can not be due to changes in NRA or in the nitrite concentration, as originally suggested (Rockel *et al.*, 2002). Rather, some other factor must limit the NO emission in the dark.



**Figure 29:** NO-emission pattern from tobacco NiR antisense mutant leaves during a dark-light-dark-nitrogen transient.

Samples (leaf discs) for NRA<sub>act</sub> and nitrite determination were taken at the time indicated (mean values, n = 3, errors bars give SD).

The extreme light-sensitivity of NO emission in Figure 29, inspite of a rather constant NRA, might indicate that NO-emission was more strongly determined by a factor derived from photosynthesis than should be expected from the minor changes in NRA and nitrite, which were originally suggested as the only determinants (Rockel *et al.*, 2002; Rockel and Kaiser, 2002). In order to distinguish between direct light effects and effects of a photosynthesis-derived metabolite on the light modulation of NO emission, leaves of clone 271 were kept in continuous light, but exposed to a transient from air to CO<sub>2</sub>-free air and back to air. NO emission dropped drastically when CO<sub>2</sub> was removed (Figure 30), although not as rapidly as during a light/dark transient (Figure 29). As before, NRA decreased only little and nitrite concentrations remained high for quite some time, confirming the conclusion that some other factor, depending on photosynthesis, would limit NR-catalyzed NO formation from nitrite and NADH. The conclusion is almost inevitable that this photosynthesis-derived factor should be, directly or indirectly, cytosolic NADH.



**Figure 30:** NO-emission from NiR-deficient transformant leaves in continuous light in the presence or absence of CO<sub>2</sub> in air.

NRA<sub>act</sub> and nitrite determination were measured at the time indicated. At T = 16 min, the light was switched on.

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## 2.4 Summarizing Conclusions

1. NO emission from leaves and cell suspensions required nitrite as substrate - there was no indication for a nitrite-independent NO production. As NR is the only source for nitrite in plants, all NO formation was finely dependent on NR.
2. If nitrite was present, some other reaction could produce NO, even in the complete absence of NR. This reaction had about the same capacity to produce NO as NR itself. It was not a MoCo-enzyme, and the reaction was cyanide-sensitive. It is not known yet whether this reaction contributes to the low NO production observed in WT plants under normal conditions.
3. High NO production required not only high [nitrite], but also either active photosynthesis (leaves), or anoxia (darkened leaves or cell suspensions). In addition to a relatively minor modulation of NRA, this is suggested to reflect the need for high cytosolic [NADH] to support high rates of NO production. Accordingly, cytosolic NADH appears to be high either under conditions of high photosynthesis, or under high glycolytic (fermentative) metabolism under anoxia.
4. In an attempt to estimate a potential NO scavenging inside cells, we compared the capacity of NR to reduce nitrate to nitrite (NRA) with its capacity to reduce nitrite to NO, *in vitro* and *in vivo*. With purified NR as a simple, low quenching system, NO emission was about 1 % of NRA. In leaves or cell suspensions, NO emission was 4 to 10 % of NRA in leaf or cell extracts. Thus, NO quenching appeared low and chemiluminescence measurement of NO emission should be a good indicator of NO production. However, direct titration experiments with cell suspensions indicated that NO quenching could be as high as 95 %. The discrepancy between these different approaches cannot be explained at present.

## CHAPTER 3

### Roles of nitric oxide under biotic stress

Among the many functions of NO in plants, its role in plant-pathogen interactions is certainly one of the most important and interesting ones (Durner and Klessig, 1999). Previous work on NO and stress responses in plants was mainly done using chemical NO donors, NO scavengers or inhibitors of NO synthesis. The number of studies that have tried to directly measure and quantify NO production under stress is very limited.

Abiotic stressors (mechanical stress by centrifugation or heat stress, among others) have been reported to stimulate NO production (Leshem *et al.*, 1998; Pedroso *et al.*, 2000; Garces *et al.*, 2001). Following pathogen attack, NO production was demonstrated in soybean or *Arabidopsis* cells (Delledonne *et al.*, 1998; Clarke *et al.*, 2000). As mentioned above, different enzymes may be responsible for NO production in plants, also during abiotic or biotic stress. A plant NOS with high homology to the mammalian enzymes has yet to be isolated. However, NOS-like activities have been detected in several plant species and notably during plant-pathogen interaction (Durner *et al.*, 1998; Foissner *et al.*, 2000; Delledonne *et al.*, 1998). More recently, a pathogen-inducible nitric oxide synthase (iNOS) in plants has been characterized as a variant of the P protein of GDC (Chandok *et al.*, 2003). On the other hand, early studies (Rockel *et al.*, 2002; Mallick *et al.*, 2000) and our results described in the second chapter prove that NO production can be mediated by NR in non-elicited conditions. Remarkably, a recent report suggested that the expression of the *NR* gene in potato was increased following infection with *Phytophthora infestans* or treatment with elicitors (Yamamoto *et al.*, 2003). However, *in vivo* evidence for the participation of NR in pathogen-induced NO production is still lacking. This lack of knowledge, and reports of Durner and co-workers (2000) prompted us to study the involvement of NR-dependent NO-emission upon treatment of tobacco with elicitors. Foissner *et al.* (2000) used DAF-2DA to monitor NO accumulation in epidermal leaf strips of tobacco treated with the fungal elicitor cryptogein, and detected a burst of NO within

minutes after addition of cryptogein. Further, NO was localized in the plastids, nuclei, plasma membrane and possibly in the peroxisomes of leaf epidermal cells.

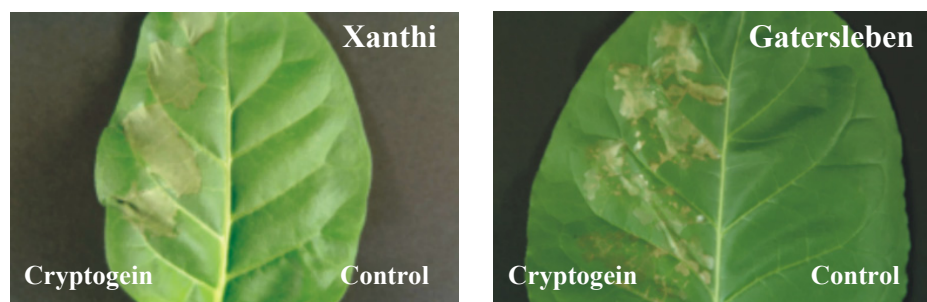
The interaction between the phytopathogenic fungus *Phytophthora cryptogea* and the non-host tobacco (*Nicotiana tabacum*) leads to a hypersensitive-type response characterised by tissue necrosis and restricted growth of the fungus, and to a systemic acquired resistance against pathogens (Bonnet *et al.*, 1986; Ricci *et al.*, 1989). At least part of that interaction appears triggered by the peptide cryptogein (10-kD), which induces numerous physiological and structural changes in tobacco. A phosphorylation/ dephosphorylation process is involved in the cryptogein reponse (Viard *et al.*, 1994; Tavernier *et al.*, 1995b) and binding studies suggested the presence of specific high-affinity binding sites for cryptogein (Kd of 2 nM) in tobacco leaf plasma membranes (Wendehenne *et al.*, 1995) and in plasma membranes from suspension-cultured tobacco cells (Bourque *et al.*, 1999). Many other reactions were reported to be induced within minutes by cryptogein: a transient and fast production of ROS by a plasma membrane NADPH oxidase (Bottin *et al.*, 1994; Viard *et al.*, 1994; Tavernier *et al.*, 1995b; Rustérucchi *et al.*, 1996; Simon-Plas *et al.*, 1997), lipoxygenase induction (Bottin *et al.*, 1994), changes in lipid composition (Tavernier *et al.*, 1995a), rapid calcium influx (Tavernier *et al.*, 1995b; Bourque *et al.*, 1998; Lecourieux *et al.*, 2002), a leakage of potassium (Blein *et al.*, 1991), cytosolic acidification (Pugin *et al.*, 1997), a rapid increase in extracellular pH and conductivity (Blein *et al.*, 1991; Pugin *et al.*, 1997), NO production (Foissner *et al.*, 2000), and early changes in gene expression (Suty *et al.*, 1995; Petitot *et al.*, 1997). Later on the time scale, cryptogein induces the production of ethylene and phytoalexins (Milat *et al.*, 1991) and finally cell death (Lebrun-Garcia *et al.*, 1998; Pugin *et al.*, 1997). Thus, interaction between cryptogein and tobacco appeared as an excellent model system for the analysing NO production and a potential role in signal transduction during the HR.

As mentioned before, most previous publications on NO production during plant-pathogen interactions favour NOS as a source for NO. Mostly, NO has been detected by using the cell permeant fluorescence indicator DAF-2DA (Foissner *et al.*, 2000). However, it is not clear at present whether NR can also have a role as a NO source in the cryptogein-tobacco interaction. Furthermore, the question of whether NO itself is necessary or sufficient for the induction of cell death, or whether it is only involved in specific reactions is still open. These questions will be examined below.

### 3.1 Cryptogein-induced programmed cell death in tobacco

#### 3.1.1 Induction of lesion formation in WT and mutant tobacco leaves

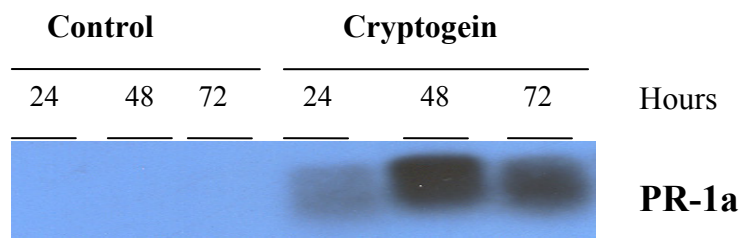
When tobacco leaves (*Nicotiana tabacum* cv. *Xanthi* and *Nicotiana tabacum* cv. *Gatersleben*) from nitrate grown-plants (attached to the plant or detached with the petiole in nutrient solution) were locally infiltrated with 10 nM cryptogein on one leaf half, they developed lesions within 16 h. After 20 to 24 h, the entire infiltrated zone was necrotized for both cultivars (Figure 31), indicating that the necrosis was independent from the cultivar. It has been reported that first, cells lose their turgidity and collapse, giving rise to small transparent areas with water- filled intercellular air spaces. The vacuolar cell volume decreased drastically (Milat *et al.*, 1991), and water loss resulted in a decreased leaf fresh weight (data not shown). The other leaf half, which was infiltrated only with buffer, remained undamaged (Figure 31). This response was time and dose-dependent; necroses developed faster in tobacco leaves when the concentration of cryptogein was increased (data not shown).



**Figure 31:** Tobacco leaves (*Nicotiana tabacum* cv. *Xanthi* and *Nicotiana tabacum* cv. *Gatersleben*) were infiltrated on the left side by the elicitor cryptogein (10 nM) and on the right side with HEPES-KOH- buffer (control). Lesion development was monitored at 24 h.

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 32 shows that cryptogein drastically increased in the expression of PR-1a, as has to be expected in a classical HR.

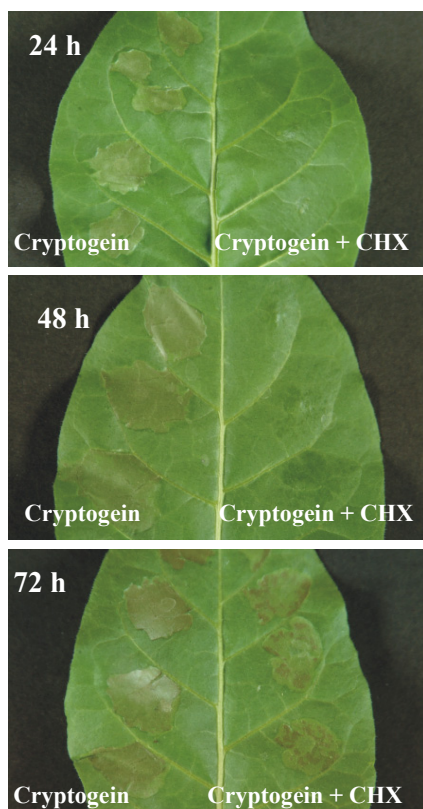




**Figure 32:** Typical time course of PR-1a expression in tobacco leaves after cryptogein treatment. Northern blot of leaf RNA isolated from tobacco subjected to infiltration with or without cryptogein. Samples are taken at different times. The row shows hybridization with [ $\alpha$ - $^{32}$ P] dCTP labeled PR-1a.

### 3.1.2 The cryptogein-induced HR requires protein synthesis

Figure 33 illustrates that cryptogein (10 nM), when co-infiltrated with CHX (200  $\mu$ M) did not induce any lesions, even over a 48 h period. Only after 72 h, cell death became slowly visible. These data demonstrate that cryptogein-induced cell death is an active process requiring *de novo* protein synthesis.

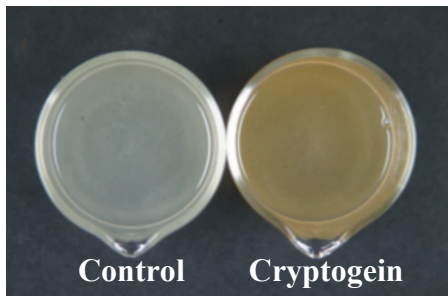


**Figure 33:** Effect of translation inhibitor on cryptogein-induced cell death on tobacco leaves.

Cryptogein (10 nM) was infiltrated in the presence or absence of cycloheximide (CHX; 200  $\mu$ M) and pictures were taken after 24 h, 48 h and 72 h.

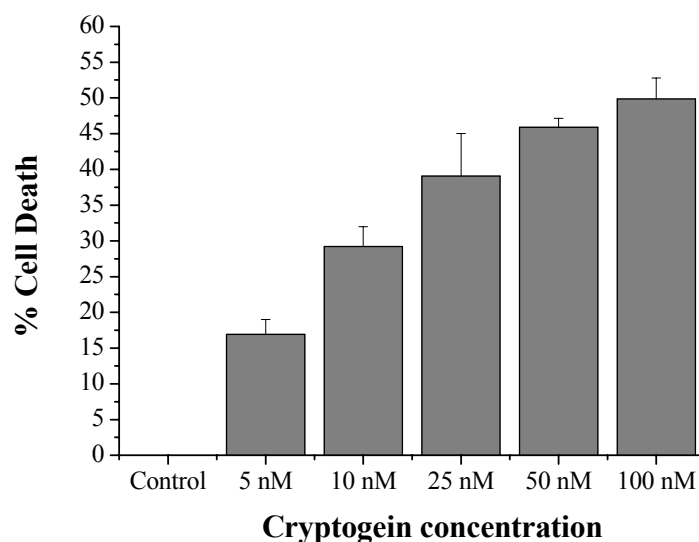
### 3.1.3 Cryptogein- induced cell death in tobacco cell suspensions

The general advantage of using cell cultures instead of intact plants has been discussed above. In context with studies on the cryptogein-induced HR, these cultures facilitate a precise dosing (in time and equal concentration and distribution) of the elicitor. Cells were established from leaves of *N. tabacum* cv. *Xanthi*. Cells exposed to cryptogein (10 nM) turned brown after a few hours (Figure 34).



**Figure 34:** Cell suspensions grown under LS medium and treated with cryptogein (10 nM) on the right side and with HEPES-KOH buffer on the left side (control). The picture was taken after 24 h.

Cryptogein-induced cell death was quantified by vital staining using Evan's blue (Levine *et al.*, 1994). Cryptogein induced the death of tobacco cells in a concentration (and dose-) dependent manner (Figure 35). In the presence of 10 nM cryptogein, about 30 % of the cells were dead after 24h.



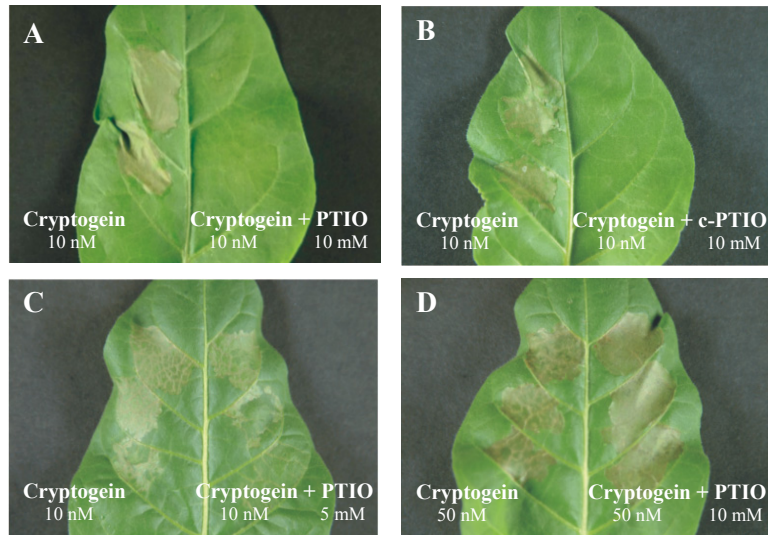
**Figure 35:** Effects of different concentrations of cryptogein (5 to 100 nM) on tobacco cell viability. The percentage of dead cells was determined after 24 h by staining with Evan's blue. Results are means ( $n = 4, \pm$  SD).

## 3.2 Participation of NO in the HR

Simultaneous production of ROS (“oxidative burst”) and of NO have been described as early events in the induction of the HR (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Bolwell, 1999; Clarke *et al.*, 2000). In the following, it is examined whether NO scavengers would prevent cell death, and whether NO production during the HR induction can be detected as NO-emission.

### 3.2.1 Effect of NO scavengers on the induction of cell death

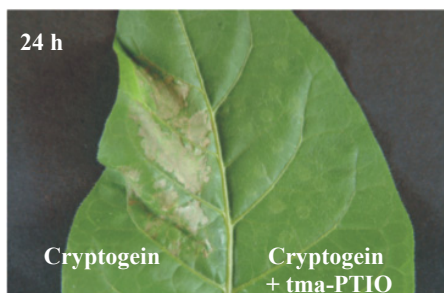
It has been show before that infiltration of leaves with cryptogein produced lesions within 24 h. Next, leaves were co-infiltrated with cryptogein and different NO scavengers. Application of 2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (PTIO) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (carboxy-PTIO; c-PTIO) (which does not react with any ROS (Barchowsky *et al.*, 1999)) has been frequently used to block NO production as well as NO-dependent cell death in tobacco, soybean, *Arabidopsis* and barley (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Foissner *et al.*, 2000; Beligni *et al.*, 2002a). When PTIO (10 mM) or c-PTIO (10 mM) was co-infiltrated with cryptogein, lesion formation was completely prevented (Figure 36A, B) or at least strongly delayed. Such a finding is usually interpreted to indicate that NO is an essential part of the signalling chain leading to the HR. However, a note of caution seems justified, since rather high concentrations of PTIO were required to prevent the HR under our conditions; lower concentrations of PTIO (less than 5 mM) were much less effective in preventing lesion formation (Figure 36C). On the other hand, higher concentrations of cryptogein (more than 50 nM) could at least partly overcome the effect of 10 mM PTIO (Figure 36D).



**Figure 36:** Effects of NO scavengers on cryptogein-induced cell death.

Leaves (from plants grown on nitrate solution) were infiltrated as indicated in the figure. Lesion development was monitored at 24 h.

While PTIO and c-PTIO are membrane permeating NO scavengers that may trap NO already inside the cells, the non-cell permeating derivative trimethylammonio-PTIO (tma-PTIO) should trap only NO that had already escaped from the cells into the apoplast. Co-infiltration of cryptogein (10 nM) with 10 mM tma-PTIO also suppressed the HR (Figure 37). Thus, up to this point the PTIO-data appeared to confirm the participation of NO in the signalling chain leading to the HR, and indicate further that trapping NO in the apoplast only was sufficient to suppress the HR.



**Figure 37:** Effect of an apoplastic NO-scavenger on cryptogein-induced cell death.

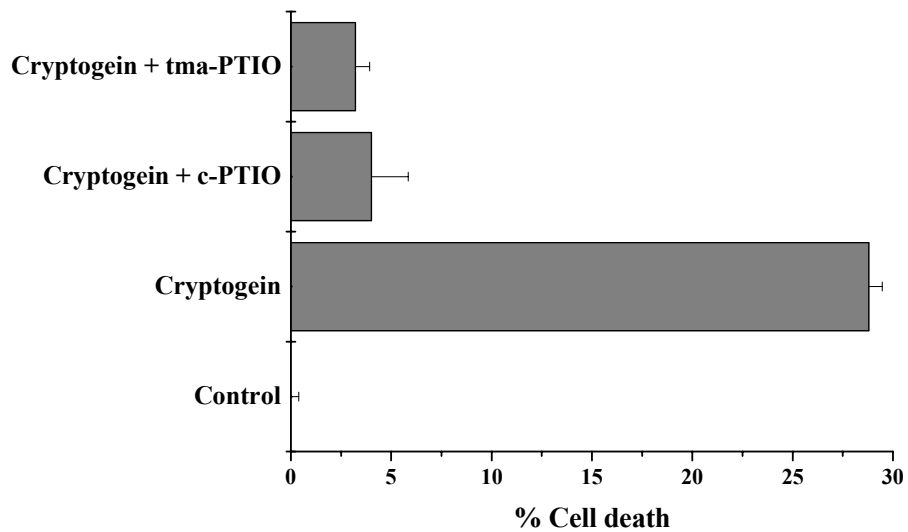
Cryptogein was 10 nM and tma-PTIO was 10 mM. Lesion development was monitored at 24 h.

It has been repeatedly stated that during the induction of the NR, NO production would be a very early event, in epidermal leaf strips within the first min after elicitor addition (Foissner *et al.*, 2000) or in soybean cell suspensions treated with *Pseudomonas* after several hours (Delledonne *et al.*, 1998). If that would also hold in our system, addition of PTIO some time after the postulated NO burst should not prevent lesion formation. This is examined in Figure 38. PTIO was infiltrated into the leaves together with cryptogein (T = 0), or 30 min (T = 30 min) or 2 h (T = 2 h) after cryptogein treatment, and lesion formation was registered after 24 h. Addition of PTIO until 30 min after cryptogein still prevented lesion formation, but addition 2 h after infiltration with cryptogein, lesion formation was less prevented (Figure 38). A conclusion would be that an NO burst within the first 2 h of elicitor addition triggers the HR.



**Figure 38:** Effect of a time delay in the infiltration of PTIO following cryptogein treatment. Left side cryptogein (10 nM), right side cyrptogein (10 nM) at T = 0 and PTIO at the times indicated. Lesion development was monitored after 24 h.

Prevention of cell death by NO scavengers of the PTIO family was also examined with cell suspensions, which permit a more precise handling of the various additions. Pre-treatment of cells (5 min before cryptogein) with the NO scavenger c-PTIO or tma-PTIO (1 mM each) reduced cryptogein induced-cell death by approximately 80 % after 24 h of treatment (Figure 39). Here again, NO production appears as a prerequisite for the HR induction.



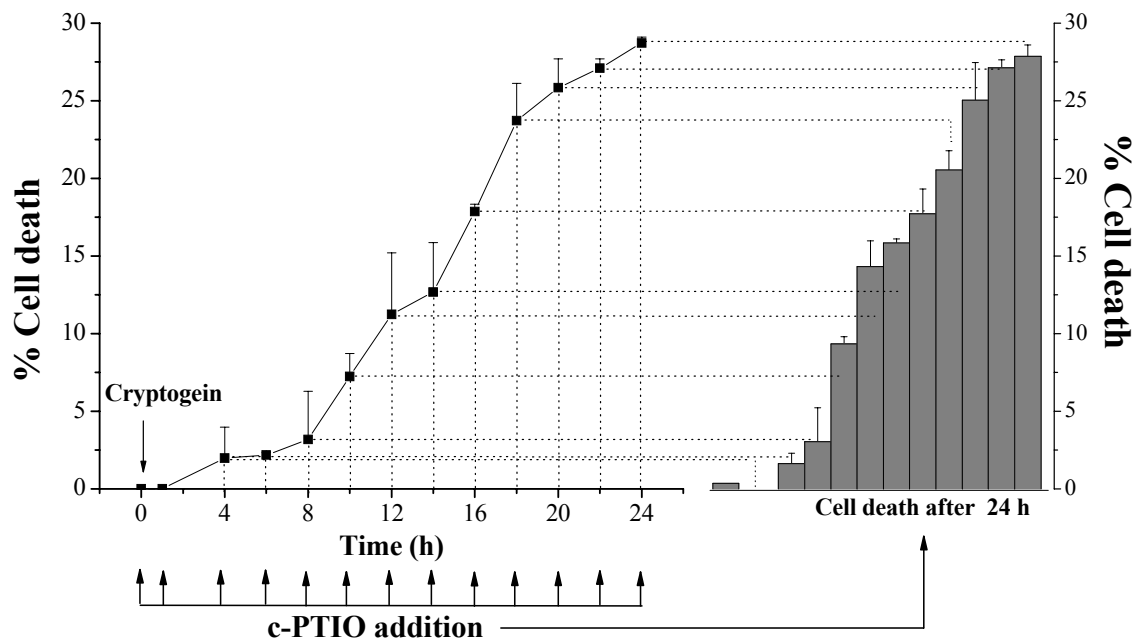
**Figure 39:** Effects of NO scavengers on cryptogein-induced cell death of tobacco suspension cultures.

Cells were incubated with 10 nM cryptogein in absence or in presence of 1 mM of c-PTIO or 1 mM tma-PTIO. The NO scavengers were added 5 min before cryptogein. The percentage of dead cells was determined after 24 h by staining with Evan's blue. Bars represent the means  $\pm$  SD of four independent experiments.

Subsequently, the 'delayed PTIO-addition' experiment was also carried out with cell suspensions. Here, however, many more time points could be taken as compared to the leaf experiment shown in Figure 40. Cryptogein was always added at  $T = 0$ , and c-PTIO was added at the indicated times after cryptogein addition. Cell viability was also examined with Evan's blue for the control (only cryptogein added at  $T = 0$ ) at various times after cryptogein addition.

Unexpectedly, the percentage of dead cells increased almost linearly (except perhaps during the first hours) with time over the whole 24 h period. There was no evidence for a 'point of no return' in the cell suspension, where the majority of cells would undergo a HR synchronously. Rather, cell death developed (or was triggered) individually in small cell fractions or perhaps even in individual cells.

In the other half of the experiment, cell viability was estimated only at 24 h after cryptogein addition, yet c-PTIO was added at the different times points were cell samples had been taken in the cryptogein control treatment. Importantly, whenever c-PTIO was added, the percentage of dead cells remained were it was before each c-PTIO addition. Thus, if c-PTIO would really prevent cell death by suppressing a short and early NO burst, the postulate would be that individual cells have to have an individual NO burst, which may occur at individual times after cryptogein addition.

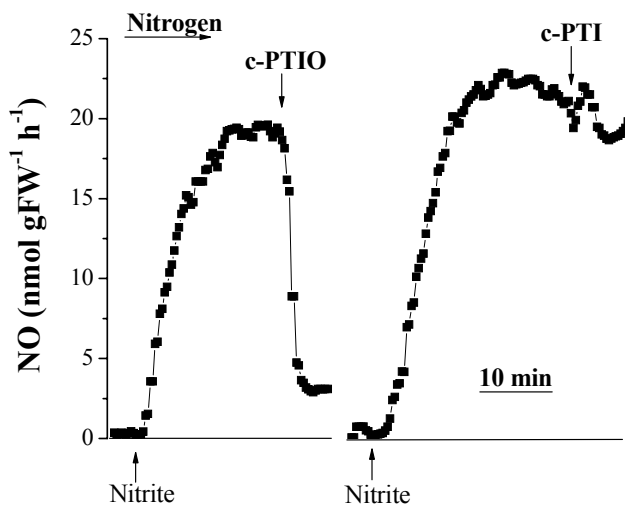


**Figure 40:** Time course of cell death of tobacco cell suspensions treated by cryptogein in absence or in presence of a NO-scavenger (c-PTIO).

In one hand, tobacco cells (0.5 mL) were incubated with cryptogein (10 nM) at T = 0 and the cell death was estimated by Evan's blue at various time points as indicated by a black point. Concurrently, c-PTIO (1 mM) was added to the cells before or at various time points, as indicated by a grey bar, after the addition of cryptogein (10 nM) at T = 0, but the cell viability was measured after 24 h ( $n = 3 \pm SD$ ).

While PTIO and c-PTIO very clearly delayed or prevented the cryptogein-induced HR, suggesting that NO was indeed required, the very high concentrations required in the leaf experiments (Figure 36A, B) prompted us to insert a control experiment. If the high PTIO

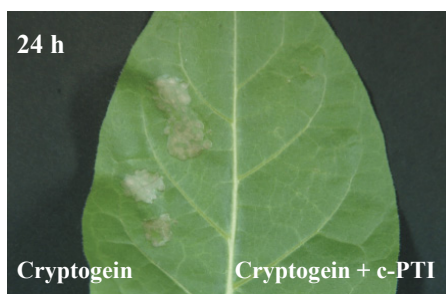
concentrations employed above would have unknown side effects, not related to their NO scavenging properties, other PTIO forms may also prevent the HR without trapping NO. When NO reacts with c-PTIO, the products of the reaction are nitrite plus c-PTI (Figure 7). In Figure 41, we first examined whether c-PTI would quench NO or not, or whether the commercial c-PTI, that was available from Dojindo Laboratories (Kumamoto, Japan), only would be free of c-PTIO. As a source for NO, we used a nitrate grown cell suspensions supplied with nitrite, under anoxia.



**Figure 41:** c-PTI and c-PTIO effects on the NO-emission from cell suspensions.

Cells were fed with nitrite (200  $\mu$ M) and flushed with nitrogen. c-PTIO and c-PTI (20  $\mu$ M) were added as indicated.

When c-PTI (10 mM) was co-infiltrated with cryptogein (10 nM) into tobacco leaves, lesion formation in the first 24 h (Figure 42) was prevented, as by c-PTIO (compare Figure 35B). Thus, the property of c-PTIO to be a specific NO-scavenger is questionable.



**Figure 42:** Effect of a c-PTI on the response to a cryptogein treatment.

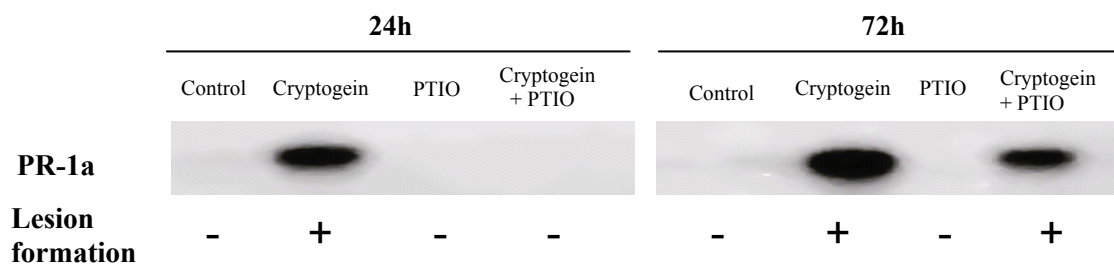
Tobacco leaves were infiltrated by cryptogein (10 nM) in absence or in presence of c-PTI (10 mM). Lesion development was monitored at 24 h. The picture represents a typical example out of four similar experiments.



### 3.2.2 Effects of NO scavengers on the expression of PR-1

Addition of NO donors to tobacco cell suspensions triggered the expression of PR-1 and other PR proteins (Durner *et al.*, 1998). Cryptogein was shown previously to induce expression of PR genes in WT plants (Figure 43). To determine whether gene activation required NO to induce cell death, PR gene expression was assayed in tobacco in response to a NO-scavenger and cryptogein. As shown in Figure 32, PTIO (10 mM) blocked accumulation of the PR-1 transcript induced by cryptogein after 24 h completely, whereas some PR-1a mRNA was detected after 72 h. It is important to notice that PTIO by itself, even after 72 h time infiltration, did not at all induce PR-1a accumulation which paralleled the late appearance of lesions.

It was then an important question whether NO would be able to induce cell death all by itself or only synergistically with ROS, as suggested by Delledonne *et al.*, 1998.



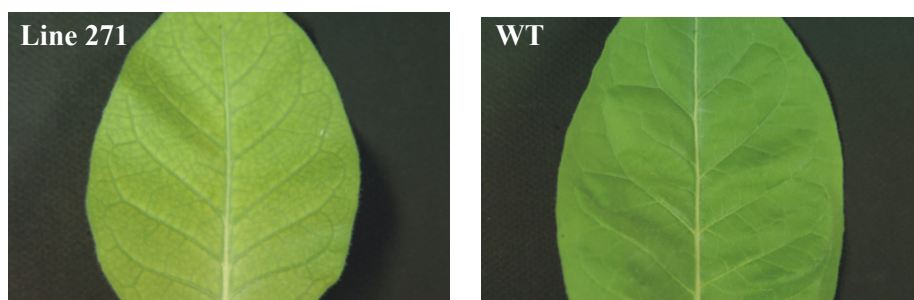
**Figure 43:** PTIO effects on the expression of PR-1a in response to cryptogein treatment.

Cryptogein (10 nM) was infiltrated into tobacco leaves together with or without 10 mM PTIO, and samples are harvested after 24 h and 72 h post infiltration. Total RNA from mock or cryptogein treated tobacco leaves was separated by electrophoresis, blotted and hybridized with PR-1a tobacco cDNA probes, as described in Materials and Methods. Lesion formation is indicated by (+) or (-).

### 3.2.3 Is NO sufficient to induce the HR?

Transgenic plants displaying only low residual levels of NiR activity constitute good tools to investigate the role of NO in plants, and especially during plant-pathogen interactions. It has been shown above that line 271, a NiR deficient mutant, grown in nitrate, exhibited a high nitrite accumulation and a concomitant NO emission in the light (Figure 29). In order

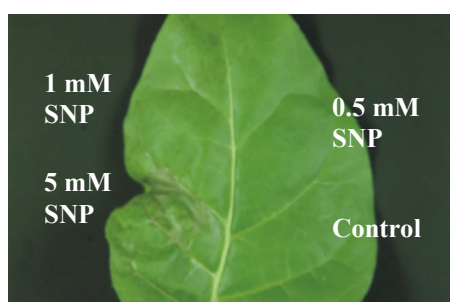
to check whether high NO production alone is sufficient to induce the HR, clone 271-plants were grown on nitrate solution during one week in a normal photoperiod. No lesions had appeared on the mutant leaves (Figure 44) even after seven days, when leaves were clearly chlorotic in comparison with the WT (*Nicotiana tabacum* cv. Gatersleben). Chlorosis was probably related to a drastic decrease of glutamine and ammonium contents in the leaf tissues of the mutant (Vaucheret *et al.*, 1992). But clearly, high levels of NO were not sufficient to trigger HR alone.



**Figure 44:** Chlorotic leaf of a NiR deficient mutant (line 271).

The transformant 271 and WT (cv. Gatersleben) were grown on nitrate solution for one week. The transformant showed chlorotic leaves, but no lesions. Pictures were taken after one week in nitrate solution. The pictures are from one typical situation that was repeated four times.

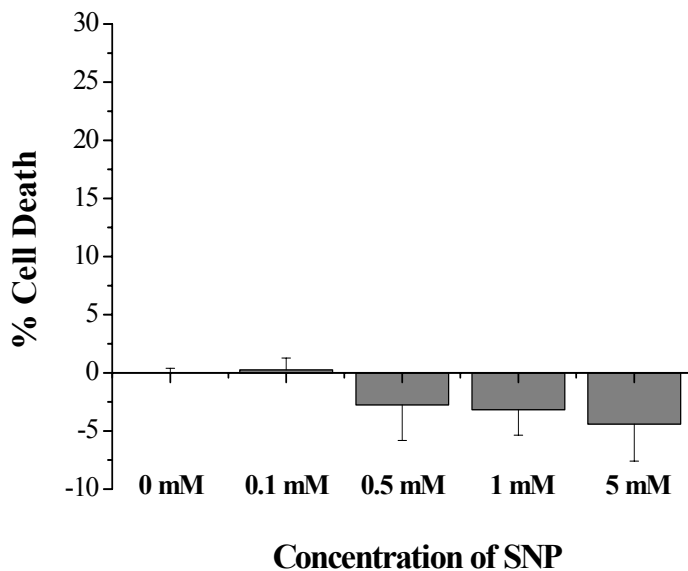
Interestingly, and in contrast to our above findings, it has been shown that elevated levels of NO produced by NO donors are sufficient to induce cell death in *Arabidopsis* cells (Clarke *et al.*, 2000) or in tobacco leaves (Huang and Knopp, 1998), without the contribution of reactive oxygen (ROS). Therefore, tobacco leaves were infiltrated with different concentrations of the NO-donor SNP. Figure 45 shows that leaf tissues infiltrated with low concentrations of SNP showed no signs of damage. Absence of lesions in *Arabidopsis* leaves treated with 0.5 mM SNP was also reported by Alamillo and Garcia-Olmedo (2001). Higher concentrations (5 mM) induced lesion-like symptoms on infiltrated tobacco tissues, but it is not clear whether that was related to NO.



**Figure 45:** Effect of SNP on induction of HR.

Leaves of tobacco plants were infiltrated with different concentrations of SNP: control (water); 0.5 mM, 1 mM and 5 mM SNP. The picture was taken 24 h after infiltration.

Treatment of soybean cell suspensions with 500  $\mu\text{M}$  SNP was estimated to generate a steady state NO concentration of about 1-2  $\mu\text{M}$  (but see discussion) which promoted the induction of cell death in the presence of ROS (Delledonne *et al.*, 1998). We therefore examined whether NO alone would induce cell death in tobacco cell suspension incubated with different concentrations of SNP in the light ( $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). SNP at concentrations between 0.1 mM to 5 mM had no effect on the viability of tobacco *cv Xanthi* cells after 24 h (Figure 46). On the contrary, the normal degree of cell death which was observed with Evan's blue after 24 h was actually abolished, confirming similar results obtained by de Pinto *et al.* (2002) with tobacco Bright-Yellow 2 cells.



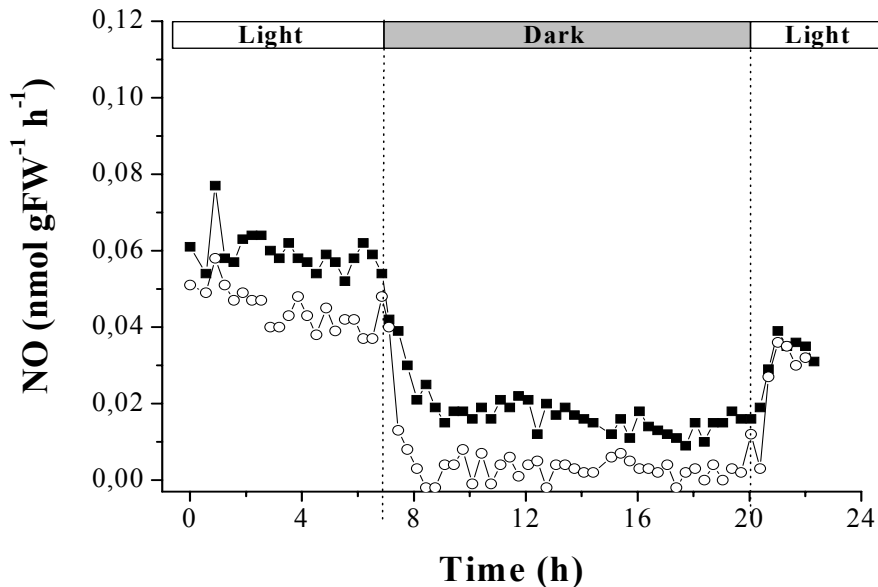
**Figure 46:** Dose-dependent response of tobacco cell suspensions treated with exogenous SNP.

The percentage of dead cells was determined after 24 h of treatment by staining with Evan's blue. Values represent mean  $\pm$  SD ( $n = 6$ ).

### 3.2.4 NO-emission during the HR

To determine whether tobacco leaves would respond to cryptogein treatment by generation of NO, as suggested by the above described effects of NO-scavengers, leaves were infiltrated with 10 nM cryptogein, and NO production was followed continuously by chemiluminescence. While leaves from nitrate-grown tobacco gave the above typical pattern of NO emission, no additional NO emission was detected from cryptogein-treated leaves during the whole phase from infiltration until lesion development (Figure 47). It

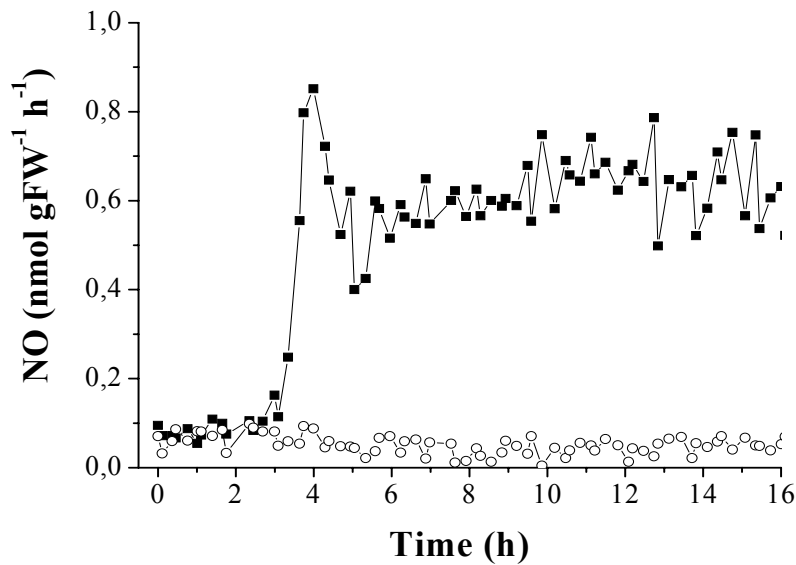
should be noted that in this figure, the “light-off peak” is missing. This is typical for a complete diurnal time course, where the light is switched off at the end of the day (This is probably due to the fact that NR is usually down regulated already towards the end of the day, approaching the same low activity as in the dark). A conclusion from these observations would be that NO plays no role in the cryptogein-induced HR, but this finding contrasts the clear protection effects by NO scavengers.



**Figure 47:** Time course of NO emission from tobacco leaves infiltrated at T = 0 with cryptogein (10 nM) (■) or with buffer (control) (○) during a 24 h diurnal cycle.

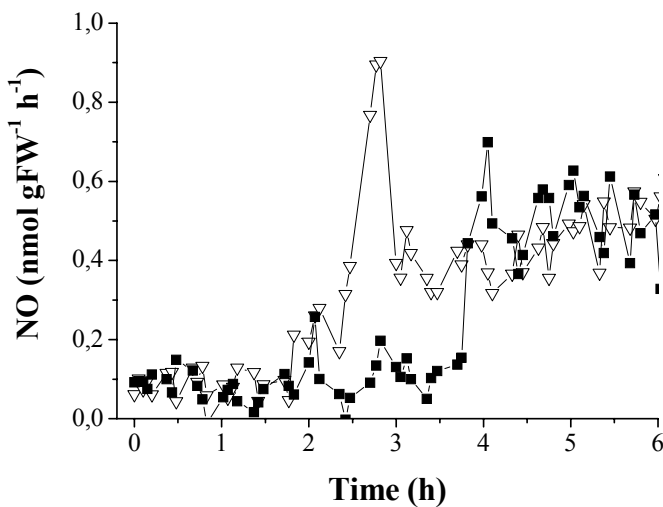
Lesion development became visible after 20h. Data represent one representative experiment out of six.

For comparison with leaves, NO-emission was also followed from a suspension of non-photoautotrophic tobacco cells before and after addition of cryptogein. Here, a very low NO emission from nitrate-grown cell suspensions could be routinely detected by chemiluminescence (Figure 48). Addition of cryptogein resulted in a slight increase of NO emission within 3 to 6 h and was maintained at a slightly higher level (0.7 nmol gFW<sup>-1</sup> h<sup>-1</sup>) than in control cells.



**Figure 48:** Time course of NO generation in tobacco cell suspensions treated with cryptogein. Cells were inoculated with 10 nM cryptogein (■) or mock-treated with HEPES-KOH (○). Data represent one experiment representative of five experiments.

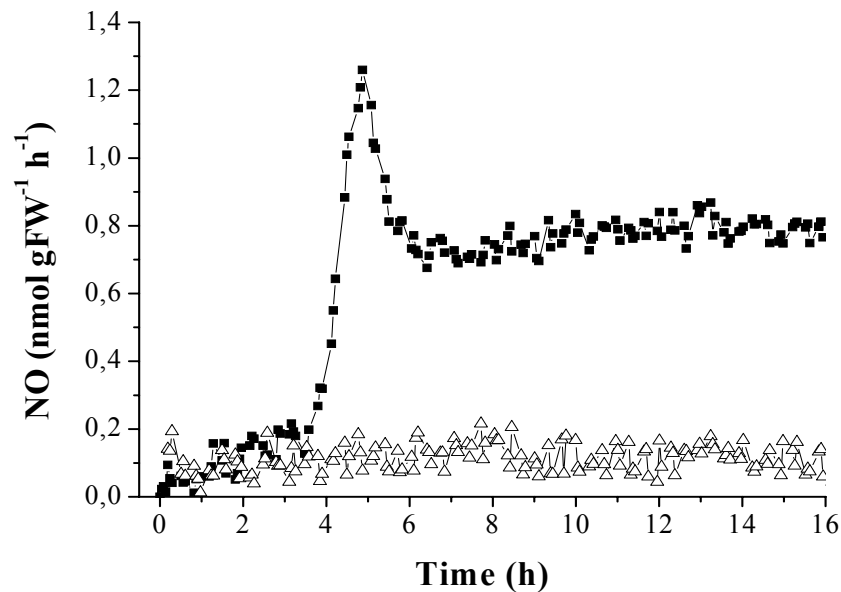
In order to understand whether the cryptogein concentration could influence the NO production, tobacco cells were again treated with 10 nM or with 100 nM cryptogein and NO emission was continuously followed (Figure 49). Clearly, the higher concentration of cryptogein (100 nM) produced an earlier but not larger increase in NO emission.



**Figure 49:** Effects on cryptogein concentration on NO-time course of tobacco cells.

Cells were inoculated with 10 nM (■) or 100 nM (▽) cryptogein at  $T = 0$ . Data represent one experiment representative of four experiments. The transient peaks were not consistent.

Pre-incubating the cells with c-PTIO (1 mM) completely suppressed the NO emission induced by cryptogein (10 nM) during HR (Figure 50).



**Figure 50:** Effects of a NO scavenger on the time course of NO generation in tobacco cell suspensions treated with cryptogein

Cryptogein (10 nM) (■) or cryptogein (10 nM) + c-PTIO (1 mM) (△) were added to the suspension, and NO production was measured. Data are from one out of three experiments.

### 3.3 Which is (are) the source(s) for NO in the cryptogein-induced cell death?

#### 3.3.1 Is NOS required for HR?

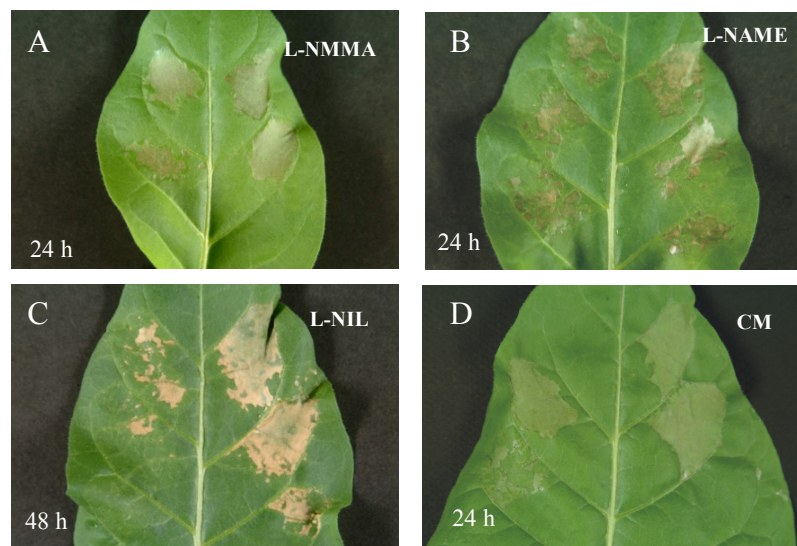
While the above experiments have indicated that NO is produced in cryptogein-elicited cell suspensions, no NO was detected with intact leaves. The reason for the discrepancy is not known at this point. But since NO scavengers were able to prevent cell death in leaves, a participation of NO could not be completely excluded. Thus, we examined for both, leaves and cell suspensions, whether NOS inhibitors could prevent cell death. Structural analogues of the NOS-substrate L-arginine, such as L-NMMA or L-NAME (N $\omega$ -nitro-L-

arginine methyl ester hydrochloride), have been used to assess participation of NOS (Ninnemann and Maier, 1996; Delledonne *et al.*, 1998; Ribiero *et al.*, 1999). The inhibitors blocked the defense response in *Arabidopsis thaliana* and the induction of programmed cell death in soybean cells treated with elicitors or pathogens (Delledonne *et al.*, 1998; Foissner *et al.*, 2000).

### 3.3.1.1 Effects of NOS inhibitors

In our hands, co-infiltration into leaves of cryptogein with L-NMMA, L-NAME or L-NIL (L-N<sup>6</sup>-(1-Iminoethyl)-lysine, acetate) (50 mM each) had either no effect on lesion development or delayed lesion formation only slightly (Figure 51A, B, C). Of course, lower concentrations of these NOS inhibitors were also tested, showing no effects on lesion formation, as expected (data not shown).

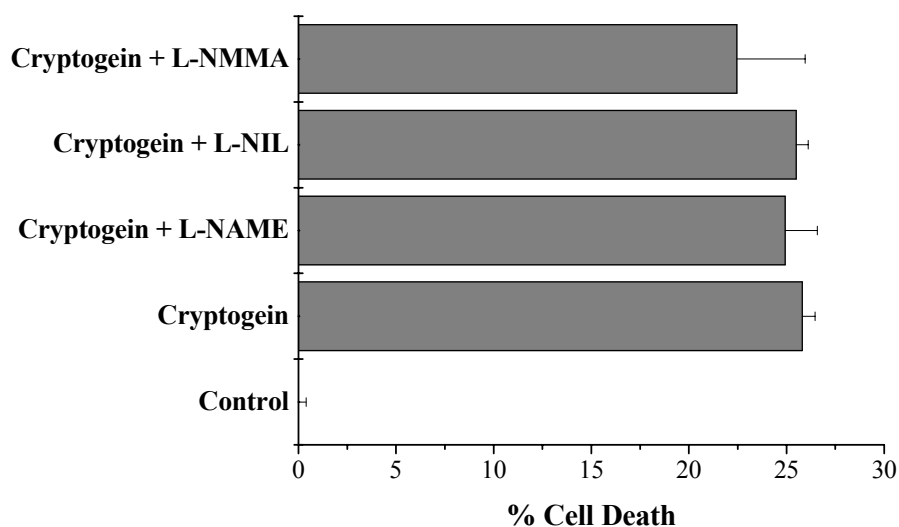
Recently, Chandok *et al.* (2003) have identified an NOS-like activity that was induced over a period of 24 h following an infection of tobacco with TMV. The NOS-like enzyme was shown to be a variant form of the P protein of the mitochondrial GDC. For GDC, inhibitors are known such as carboxymethoxylamine (CM). When our tobacco leaves were co-infiltrated with cryptogein (10 nM) and CM (20 mM), cell death was not prevented (Figure 51D).



**Figure 51:** Effects of NOS inhibitors and GDC inhibitor on cryptogein induced cell death.

Tobacco leaves were co-infiltrated with cryptogein (10 nM) and L-Arg (50 mM) on the left side and on the right side with L-NMMA (A), L-NAME (B) and L-NIL (C) (50 mM each) and CM (D) (20 mM). The pictures were taken at time as indicated.

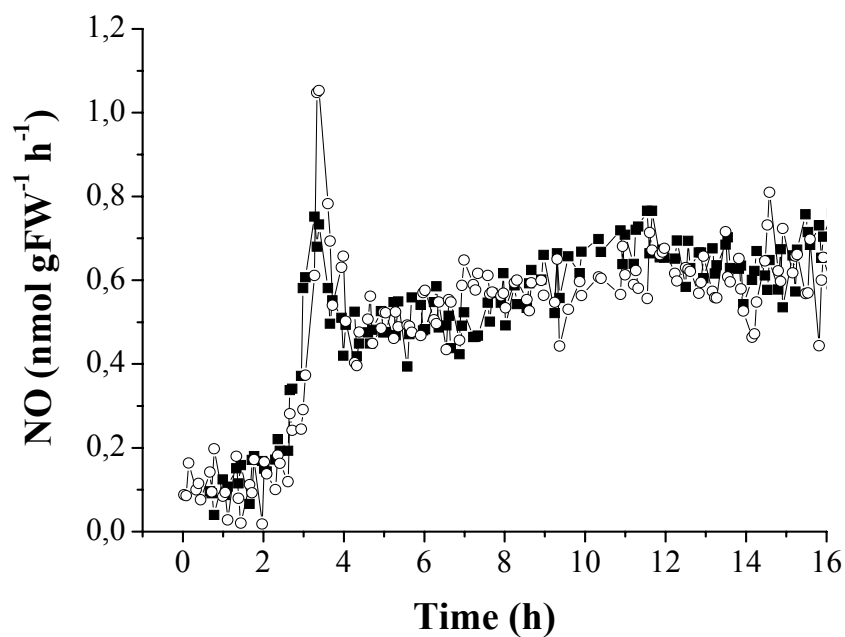
The effects of potential NOS inhibitors on the viability of the tobacco cell suspensions were also determined. Cells were exposed to cryptogein (10 nM) during 24 h in the presence of either L-NMMA, L-NAME or L-NIL, three inhibitors of mammalian NOS, at 2 mM final concentration (Figure 52). Neither compound could prevent cell death induced by cryptogein.



**Figure 52:** Cell death of cultured tobacco cells treated with cryptogein and NOS inhibitors. Cells were treated with cryptogein (10 nM) in the absence or presence of L-NMMA or L-NIL or L-NAME (2 mM each) or with H<sub>2</sub>O (control). Cell viability was estimated by Evan's blue staining after 24 h. Bars represent the means  $\pm$  SD of four independent experiments.

Although these inhibitors did not prevent cell death, a role of NOS as a source of NO in cryptogein-treated cell suspensions (see Figure 52) was investigated. Addition of L-NMMA, at final concentrations of 2 mM, together with cryptogein to the cell culture, did not interfere with NO-production (Figure 53). Similar results were obtained with other NOS inhibitors (L-NIL and L-NAME) (data not shown).



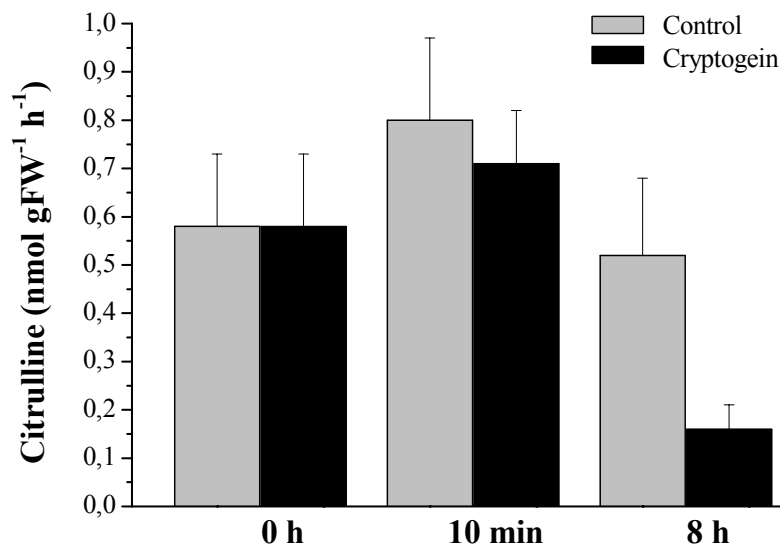


**Figure 53:** Effects of NOS inhibitors on NO-generation induced by cryptogein in tobacco cell suspensions.

Tobacco cells were placed into a transparent lid chamber, mounted on a shaker. The cells were treated at  $T = 0$  by cryptogein (10 nM) (■) or cryptogein (10 nM) + L-NMMA (2 mM) (○). Data represent one experiment representative of three experiments.

### 3.3.1.2 NOS activity in leaves after cryptogein treatment

In spite of a lack of an effect of NOS inhibitors, a potential NOS activity was also checked directly by following the conversion of  $^3\text{H}$ -labeled arginine into citrulline in crude extracts from leaves with or without cryptogein pre-treatment (Figure 54). A low NOS-like activity was detected in control leaves, but this activity was not increased by cryptogein pretreatment of the leaves; in contrary, NOS activity was even decreased. This NOS activity was similar to the basal NOS activity found in tobacco before infection with TMV (Durner *et al.*, 1998; Chandok *et al.*, 2003). However, even *in vitro*, this apparent NOS activity was only weakly sensitive to 1 mM L-NMMA (36.6 % inhibition). Under the same conditions, the activity of a commercial constitutive neuronal NOS from rat brain (Calbiochem, Schwalbach, Germany) was completely blocked (98.5 % inhibition) (data not shown).

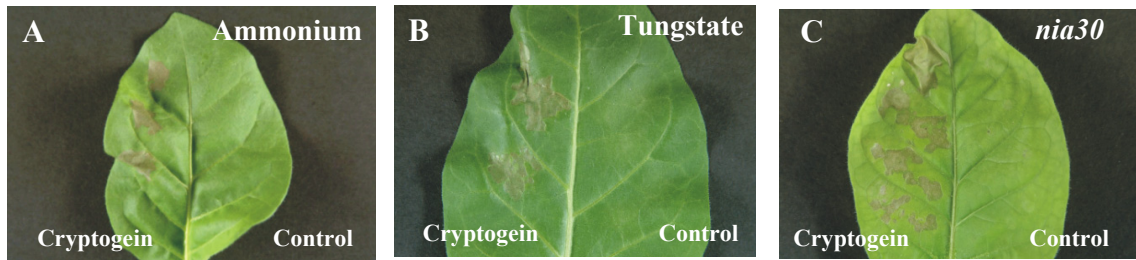


**Figure 54:** A NOS-like activity in extracts of control or cryptogein treated tobacco leaves. After infiltration with cryptogein (10 nM) or buffer (control) at T = 0, extracts were prepared at the indicated times, and NOS activity was measured by the <sup>3</sup>H-arginine/citrulline assay. Results are means of 6 independent measurements  $\pm$  SD. For further details see Materials and Methods.

### 3.3.2 *Is nitrate reductase required for the HR?*

According to the above results, the role of NO in the PCD seemed still unclear; the results with NO scavengers, and the fact that NO was produced in response to cryptogein by cell suspensions, would actually support such a role, but the above studies with NOS inhibitors as well as direct measurements of NOS activity in leaf extracts contradict it. Accordingly, some other source for NO would be needed, and NR would certainly be a candidate with high capacity. To our knowledge, here is as yet only one paper suggesting a role of NR in plant-pathogen interactions (Yamamoto *et al.*, 2003). Therefore, we examined whether NR could be a source for NO during cryptogein-induced cell death. We used plants totally devoid of NRA: 1) leaves from ammonium-grown plants, 2) leaves from WT plants that were cultivated on a medium containing 500  $\mu$ M sodium tungstate instead of molybdate, or 3) leaves of the NR-free *nia30* double mutant. With all these plant types, infiltration of cryptogein induced normal lesion development (Figure 55A, B). Only with the *nia30*

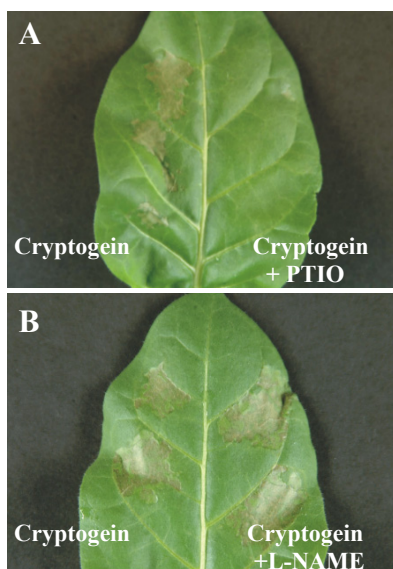
mutants, lesion development was slower than in WT (Figure 55C), which was probably due to the very slow growth of these plants.



**Figure 55:** Response to cryptogein of NR-free leaves: ammonium grown (A), tungstate-treated (B), NR-free *nia30* mutant (C).

Tobacco leaves totally devoid of NR were infiltrated with buffer (control) or with cryptogein (10 nM). The lesion development was monitored after 24 h (A, B) and 72 h (C).

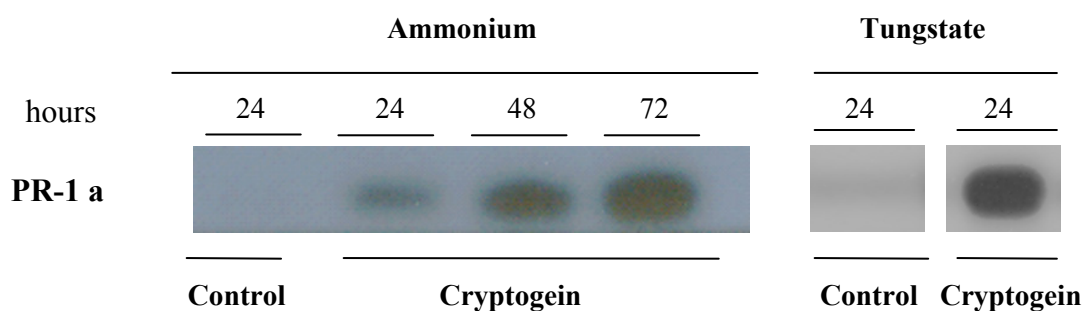
Of course, all these experiments could not be completely exclude that yet another unknown source for pathogen-induced NO might exist in plants, in addition to or instead of NR or NOS. It seemed reasonable, therefore, to check whether NO scavengers would also prevent lesion formation in these NR-free plants. Figure 56A shows again that lesion formation induced by cryptogein (10 nM) was prevented by PTIO. An involvement of NOS was also tested in ammonium-grown tobacco leaves by infiltrating L-NAME (50 mM) with cryptogein (10 nM) (Figure 56B). As before, this inhibitor had no effect.



**Figure 56:** Effects of an NO-scavenger and a NOS inhibitor on the induction of cell death by cryptogein in NR-free, ammonium-grown tobacco leaves.

Leaves were treated with cryptogein (10 nM) in absence or in presence of PTIO (10 mM) (A) or L-NAME (50 mM). The pictures were taken 24 h after infiltration.

PR-1 expression was also examined in the above NR-free plants (Figure 57). Treatment of ammonium leaves with 10 nM cryptogein resulted in the appearance of PR-1a mRNA after 24 h, with increased accumulation continuing up to 72 h. Similar PR-1a expression was observed after 24 h treatment with cryptogein in tungstate-treated leaves.



**Figure 57:** Induction of PR-1a transcripts in cryptogein-treated, NR free tobacco plants which were grown on ammonium or on tungstate.

Sampling times after cryptogein treatment (at T = 0) are indicated. Hybridisation was done with a [ $\alpha$ - $^{32}$ P] dCTP labeled PR-1a probe, and equal amounts of total mRNA were spotted.

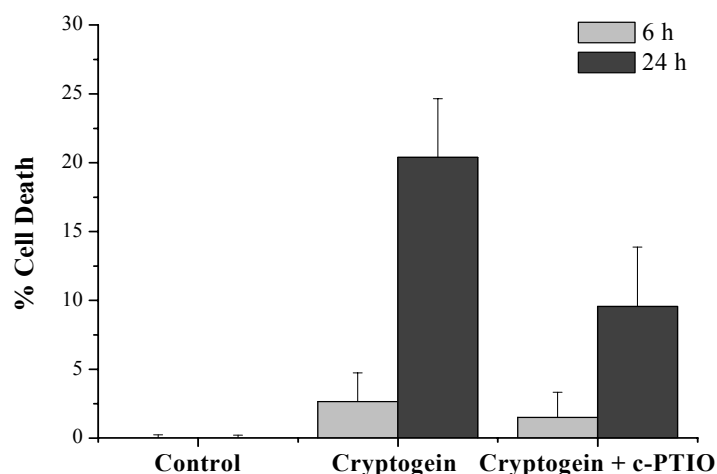
The non-involvement of NR in the induction of HR was confirmed using ammonium-grown tobacco cells treated with cryptogein. As shown in the Figure 58, ammonium cells turned brown after few hours, just like normal, nitrate grown cells (Figure 34).



**Figure 58:** Ammonium cell suspensions treated with cryptogein (10 nM) or with HEPES-KOH buffer (control).

The cells were continuously agitated on a rotary shaker (100 rpm). The picture was taken at 24 h after elicitation.

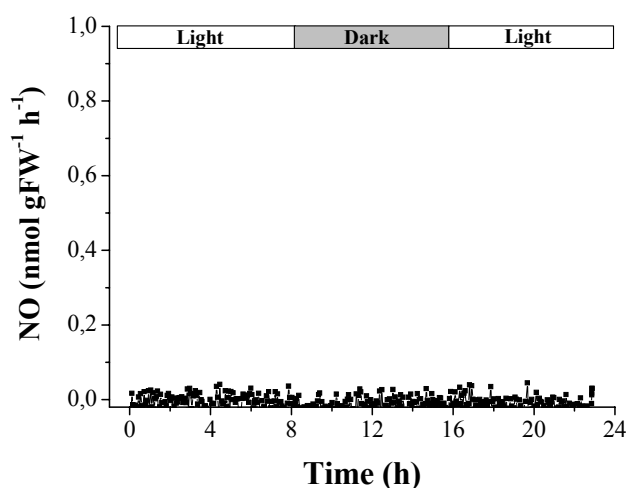
Induction of death of ammonium-grown cells by cryptogein in presence or absence of c-PTIO was depicted in the Figure 59. After 6 h, the percentage of dead cells was only slightly above zero (non-significant) for both treatments. After 24 h, around 20 % of cell had died, whereas with c-PTIO it was only 10 %.



**Figure 59:** Cryptogein-induced cell death in ammonium-grown tobacco cell suspensions.

Cells were incubated in absence or in presence of 10 nM cryptogein with and without c-PTIO (1 mM). Viability of the cells was determined by Evan's blue staining. Data bars represent the mean ( $n = 3$ )  $\pm$  SD.

As shown above, all NR-free leaves (by whatever treatment), which showed no NO-emission under normal conditions (Table I, II; Figure 26) but which developed the HR, also gave no additional NO emission following cryptogein treatment (Figure 60). Ammonium and tungstate-grown cell suspensions were also investigated. These results were similar to these obtained with NR-free leaves treated with cryptogein (compare Figure 60).



**Figure 60:** Time-course of NO-emission from NR-free leaves after cryptogein treatment.

Conditions are as in Figure 47.

Data are mean ( $n = 5$ ).

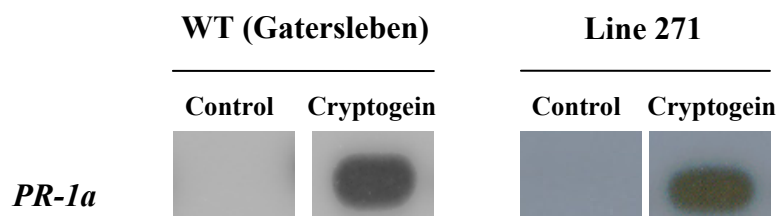
### 3.4 Effect of an overproduction of NO on the development of the cell death

It has been suggested that ROS ( $O_2^-$ ,  $H_2O_2$ ) and NO have to be co-produced in stoichiometric quantities during the HR, and that an excess of one over the other would also weaken the HR (Delledonne *et al.*, 2001). In order to verify this assumption, we examined lesion formation of NiR antisense transformants (clone 271), which always emit high amounts of NO when grown on nitrate (compare Figure 29). Upon cryptogein treatment, these plants produced normal lesions visible 16 h after infiltration (Figure 61). No symptoms were observed in HEPES-KOH-infiltrated leaves.



**Figure 61:** Leaf from a NiR antisense-transformant (*N. tabacum* cv Gatersleben) was infiltrated with 10 nM cryptogein in the left side and with HEPES-KOH buffer in the right side (control). The picture was taken after 24 h.

The expression of PR-1a was also investigated in these transformant leaves (Figure 62). After 24 h, WT and mutant expressed PR-1a, showing that an overproduction of NO did not interfere in the induction of defense gene.



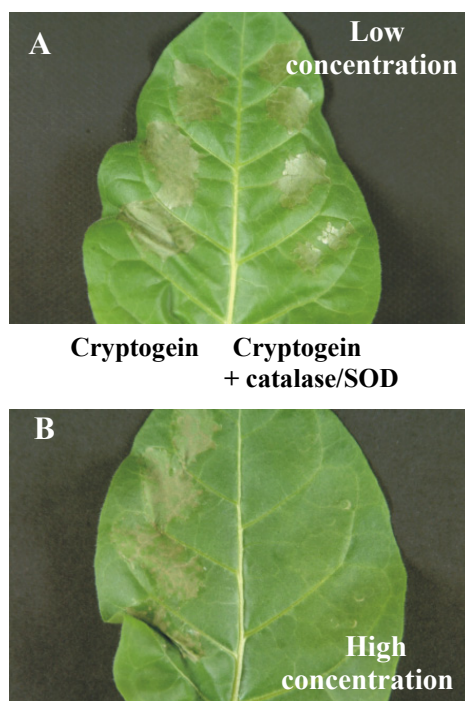
**Figure 62:** PR-1a expression in tobacco WT (*Nicotiana tabacum* cv. Gatersleben) and NiR deficient mutant leaves after cryptogein treatment.

Samples are taken at 24 h. Other conditions are as in the Figure 57.

### 3.5 On the role of ROS in the cryptogein-induced cell death, and their possible interaction with NO

It has been reported that NO and ROS have different effects depending on whether they are separately or simultaneously produced in tobacco cells (de Pinto *et al.*, 2002). During HR, an early event could be transient formation of ROS, either in parallel with or followed by a transient NO-production (Durner *et al.*, 1998). On the other hand, it has been above shown that some quenching of NO from purified NR by endogenous superoxide or H<sub>2</sub>O<sub>2</sub> could occur. Thus it seemed possible that the detection of NO emission from cryptogein-treated leaves was impaired because of a parallel production of ROS. An oxidative burst triggered by cryptogein was reported to be rapid (within minutes) and transient (Viard *et al.*, 1994; Rustérucci *et al.*, 1996; Pugin *et al.*, 1997; Simons-Plas *et al.*, 1997).

In order to remove ROS potentially formed in response to cryptogein, antioxidant enzymes (SOD and catalase) were co-infiltrated with cryptogein (10 nM). Unexpectedly, when the concentration of antioxidant enzymes was 20 U mL<sup>-1</sup> (each), these leaves responded to cryptogein with normal lesion development (Figure 63A). Only if the concentrations of these enzymes were 300 and 2000 U/mL for SOD and catalase, respectively, lesions were totally prevented (Figure 63B).

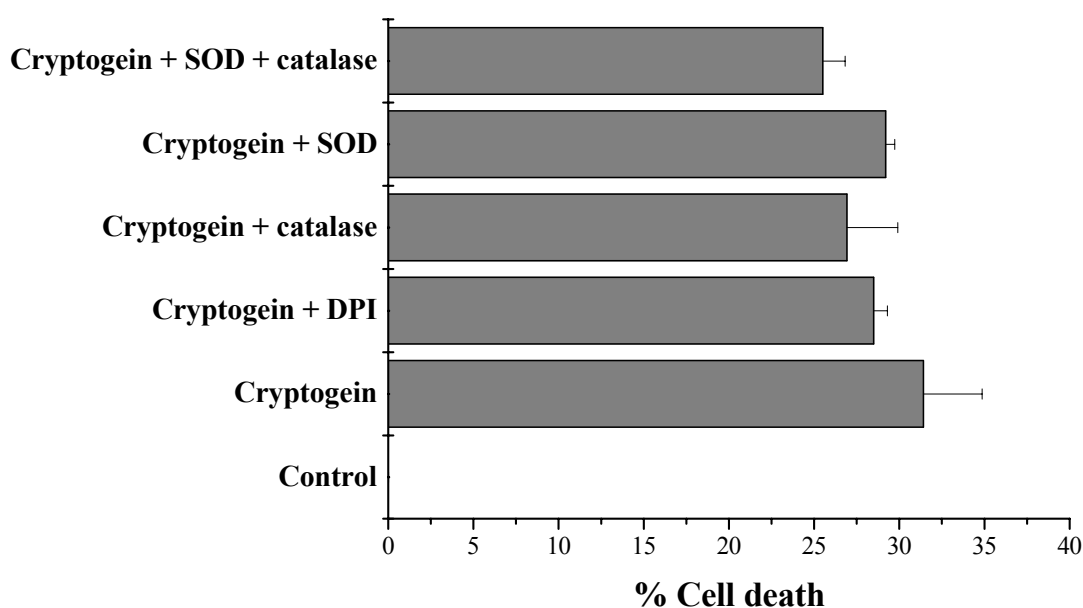


**Figure 63:** Effect of antioxidant enzymes on cryptogein induced cell death.

Cryptogein (10 nM) was infiltrated in the absence or in presence of low concentrations of SOD and catalase (20 U mL<sup>-1</sup>, each) (A) or high concentrations of SOD (300 U mL<sup>-1</sup>) and catalase (2000 U mL<sup>-1</sup>) (B). The pictures were taken after 24 h.

The very high concentrations of antioxidant enzymes required to prevent HR make the interpretation difficult. Basically it appears that ROS are somehow required in the HR. It is important to note that high concentrations of catalase (a heme-protein) may not only reduce the concentration of ROS, but may also react with NO present in the tissues. But as before, absolutely no additional NO-emission induced by cryptogein could be detected (data not shown) (compare Figure 47) in leaves co-infiltrated with cryptogein and low concentrations of SOD and catalase.

To further elucidate the relation between ROS and cell death in cryptogein-treated tobacco cells, the effect of the NADPH oxidase inhibitor DPI, which prevents the production of  $O_2^-$ , and of ROS scavengers (SOD and catalase) on the cryptogein induced cell death were also investigated with cell suspensions (Figure 64). None of them had an effect on cell death. Based on these results, it appears that the induction of cell death by cryptogein did not require the production and accumulation of  $O_2^-$  and  $H_2O_2$ .

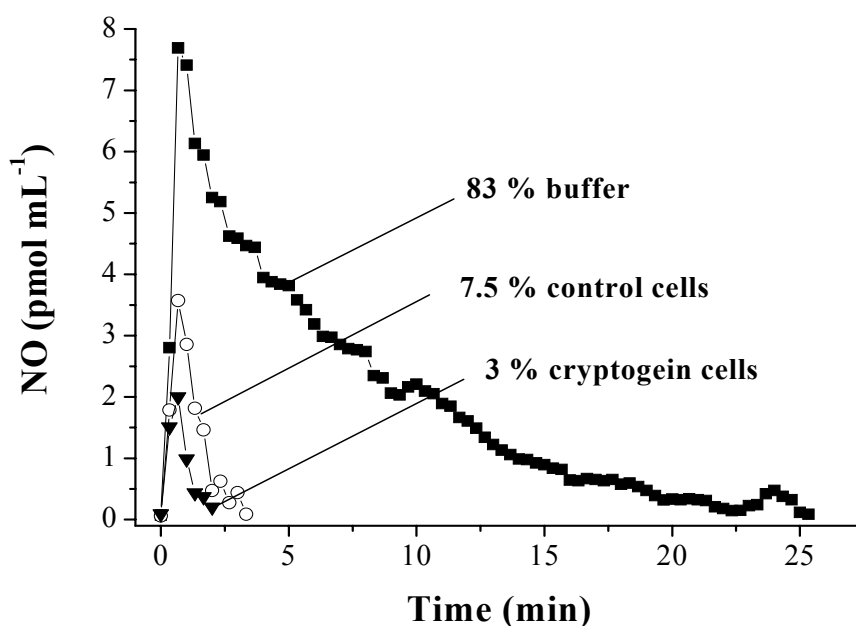


**Figure 64:** Effects of an inhibitor of ROS production and of ROS-degrading enzymes on cryptogein-induced cell death in cell suspensions.

DPI ( $1 \mu\text{M}$ ), SOD ( $200 \text{ U mL}^{-1}$ ) and catalase ( $2000 \text{ U mL}^{-1}$ ) were added 5 min prior to cryptogein treatment ( $10 \text{ nM}$ ). After incubation for 24 h, cell death was monitored by staining with Evan's blue. Bars represent the means  $\pm$  SD of three independent experiments.



As shown in Chapter 1, NO may react rapidly with living cells. Biological NO scavenging, however, may not be constant, but depend on the type and status of the cells. As mentioned in the introduction, during the HR, an early event would be transient formation of ROS, either in parallel with or followed by a transient NO-production (Durner *et al.*, 1998). ROS are known to react rapidly with NO to form N-compounds of higher oxidation states, and might therefore act as most efficient scavengers for NO, preventing or decreasing emission from the leaves or cell suspensions and detection by chemiluminescence. The same type of experiment shown in chapter 1 (Figure 11 and 12), was carried out with cryptogein treated cells. During 24 h, treated cells (10 nM cryptogein) and their control cells were agitated on a rotary shaker at 100 rpm under continuous light (24°C). These cells were used to compare NO quenching of cryptogein-treated and of control cells. NO was added as a solution prepared by flushing NO in nitrogen (100 ppm) through water (for details compare Figure 11). Figure 65 shows that NO quenching was indeed slightly higher in cells pre-treated with cryptogein than in untreated cells.



**Figure 65:** Effect of cryptogein-treatment on NO scavenging of a tobacco cell suspension.

A- Two mL of the solution, prepared as described above, were injected into 1 mL of buffer solution (■), into 1 mL of a tobacco cell suspension (○) or into 1 mL of cells treated with 10 nM cryptogein for 24 h (▼). Cells were incubated in a small Petri dish placed in a glass cuvette, mounted on a shaker. Each curve represents the mean of five replicate experiments. The numbers at the curves indicated the amount of NO that was recovered (as % of the total added NO). If the percentage is small, this would indicate heavy quenching.

### 3.6 Summarizing conclusions

The fungal peptide cryptogein was used as an elicitor of the hypersensitive response in tobacco leaves and cell suspensions. Cryptogein even at a low concentration (10 nM) reliably induced cell death and triggered the expression of PR-1 when infiltrated into tobacco leaves or added to cell suspensions. The HR could be prevented by NO-scavengers (c-PTIO), which would indicate that NO production was indeed required for the HR. However, no cryptogein-induced NO emission could be detected. Further, the HR was independent of NR, and was also insensitive to NOS inhibitors, suggesting that NO was actually not required. The obvious and severe contradiction cannot be sufficiently solved. Since the product of the reaction of c-PTIO with NO, c-PTI, also prevented the HR, but without quenching NO, it seems most probable that c-PTIO may have prevented the HR by as yet unknown side effects, not related to NO quenching. In that case, the final conclusion would be the NO plays no role in the cryptogein-induced HR.

The fact that cryptogein induced a NO emission from nitrate grown cell suspensions, but not from ammonium cells, is not in contradiction to the above opinion. In a recent paper, Yamamoto *et al.* (2003) found an induction of NR transcripts by a fungal cell wall elicitor. Thus, increased NO production may be a consequence of the HR induction, not a prerequisite.

Our experiments with the NO overproducing clone 271 did not confirm the recent proposal by Delledonne *et al.* (2001), that NO and ROS have to be produced in stoichiometric amounts in order to trigger PCD.

## C DISCUSSION

### 1 Rates and limiting factors of NO emission

The NO-measurement by chemiluminescence carried out with tobacco plants and cell suspensions under non stress-conditions confirmed previous results (Rockel *et al.*, 2002; Morot-Gaudry-Talarmain *et al.*, 2002) that NO is produced by NR from nitrite and NADH. Rates of NO emission from normal leaves were rather low, usually below 1/1000 of NR activity, or, in absolute terms, in the range of 0.20 nmoles gFW<sup>-1</sup> h<sup>-1</sup>. In these leaves, nitrite concentrations and NO emission rates were higher in the light than in the dark, and both were much higher (up to 1000 fold) under nitrogen (dark). Thus, NO emission appeared strictly correlated with the nitrite concentration. While this is certainly true, our results suggest that some additional factor might limit NO emission, at least under conditions of optimal nitrite supply. Under anoxia, nitrite reduction stops while NRA is increased (compare Botrel *et al.*, 1996; Botrel and Kaiser, 1997) which causes high tissue nitrite concentrations; but at the same time anoxia is supposed to create high NADH/NAD<sup>+</sup> ratios due to high glycolytic fluxes, a situation which is known to result in fermentation as a mean to recycle NAD<sup>+</sup>. Thus, the high cytosolic NADH may be an additional factor favouring high NO emission from anoxic tissues.

This view is further supported by our experiments with transgenic tobacco plants expressing an antisense NiR construct (Clone 271; Vaucheret *et al.*, 1992). These plants have very low NiR but normal NR activities. Accordingly, they accumulated high nitrite concentrations. Photosynthesis drastically stimulated this nitrite accumulation, and accordingly, NO emission was at maximum in the light. Originally it was suggested that the very high NO emission from these leaves (up to 100 times higher than from WT) was again exclusively dictated by the nitrite concentration, and that the higher nitrite accumulation in light as compared to dark would simply reflect the NR activation state (in the absence of NiR) (Morot-Gaudry-Talarmain *et al.*, 2002). However, NO emission dropped drastically when leaves of clone 271 were transferred from light to dark in air, or from air to CO<sub>2</sub>-free air in continuous light. In both cases, photosynthesis stopped, yet nitrite and NRA remained high for some time. The conclusion is inevitable that

photosynthesis provided “something” that helped to maintain high NO emission rates. We suggest cytosolic NADH to be the factor. A similar situation was created when cell suspensions, supplied with sufficient nitrite, were transferred from anoxia to air: NO emission dropped, while nitrite and NRA remained high. Here again, we suggest that NADH was high under anoxia and low in air, which is a common situation normally driving fermentation. All together, it seems a reasonable assumption that at high nitrite supply, NADH may strongly limit NO production in non-photosynthetic tissues in air, or in green tissues in the dark. The same has previously been concluded for reduction of nitrate to nitrite (compare Kaiser *et al.*, 2000; Kaiser *et al.*, 2002a). Unfortunately, it seems almost impossible to measure cytosolic free NADH concentrations directly (Igamberdiev and Gardeström, 2003).

## 2 NO emission = NO production?

While ozone-mediated NO-chemiluminescence is an extremely sensitive (down to the ppt-range) and specific method for real-time NO quantification, it has a basic disadvantage shared with some other NO detection systems like EPR, NO-electrodes or the hemoglobin method: it detects only the NO that has been released from the cell or plants, and much of the produced NO may actually react inside, thereby escaping detection. Therefore, the question, “does NO-emission reflect NO-production?” seems to be an important point to be clarified. We used different approaches to estimate a possible NO quenching.

**Approach A:** It is based on a comparison of the ratio of NO emission versus NRA, both at substrate saturation, *in vitro* and *in vivo*. First, we measured NO emission from a solution of highly purified NR at nitrite and NADH saturation, and NRA at nitrate and NADH saturation, assuming that such a simple solution has a very low NO quenching capacity. Next, we measured NO emission from whole leaves or cell suspensions at nitrite saturation *e.g.* under nitrogen or in the light (= high NADH). Nitrite saturation in leaves was obtained by using the NiR-deficient mutant clone 271, and in cell suspensions saturating nitrite was added. Subsequently, NRA was measured in a crude leaf or cell extract under NADH and nitrate saturation (+ Mg<sup>2+</sup>). If NO quenching *in vivo* would be high, the apparent NO emission as percent of NRA should be much lower than with purified NR. With highly

purified NR solution, the rate of NO-emission was 0.80 % of NRA (Figure 17). Under nitrogen, the rate was slightly above 1 %. This percentage is consistent with data from Rockel *et al.* (2002) and Yamasaki and Sakihama (2000). The small difference between air and nitrogen in NO emission by purified NR is probably related to the fact the NR can produce  $O_2^-$  (Barber and Kay, 1996) and consequently, to form  $ONOO^-$  by combination of NO and  $O_2^-$  (Yamasaki *et al.*, 1999). But it has also been reported that the NO production rate by NR may be as high as the rate of nitrate reduction (Dean and Harper, 1988; Klepper, 1990). The reason for this big discrepancy is not clear, but it may simply reflect the technical difficulties in quantifying NO.

The *in-vivo* NO-emission by leaves of clone 271 (air, light) was 4.15 % of the NRA. With cell suspensions fed with nitrite, similar results were obtained under nitrogen (NO = 2-5 % of NRA). Thus, in both cases the NO emission relative to NRA was higher, not lower than with purified NR. Accordingly, it seems very improbable that NO scavenging in the leaves was high, and the conclusion would be that gas phase chemiluminescence properly reflects NO production rates. The fact that the NO/NRA ratio was occasionally higher than with purified NR may be due to difficulties in determining the real NRA, or it may be caused by other reactions, aside of NR, that would contribute to NO production from nitrite and NADH (see below).

**Approach B:** Titration of cell suspensions with NO solutions. When a solution with known NO concentration was injected into pure water, the total amount of NO emitted from water into the air was almost exactly as calculated from the Henry coefficient, indicating that NO quenching was negligible. In contrast, only a small part (5 to 15 %) of the added NO was emitted from cell suspensions, indicating that 85- to 95 % of the added NO had been trapped by the cells. About 50 % of that quench was not due to the cells themselves, but to the complex cell medium. This rather large NO scavenging is in marked contrast to the results from approach A. The only explanation is that injection of NO caused a rather high peak concentration of NO, which favored rapid reaction of NO with cellular material.

### 3 Which reactions produce NO?

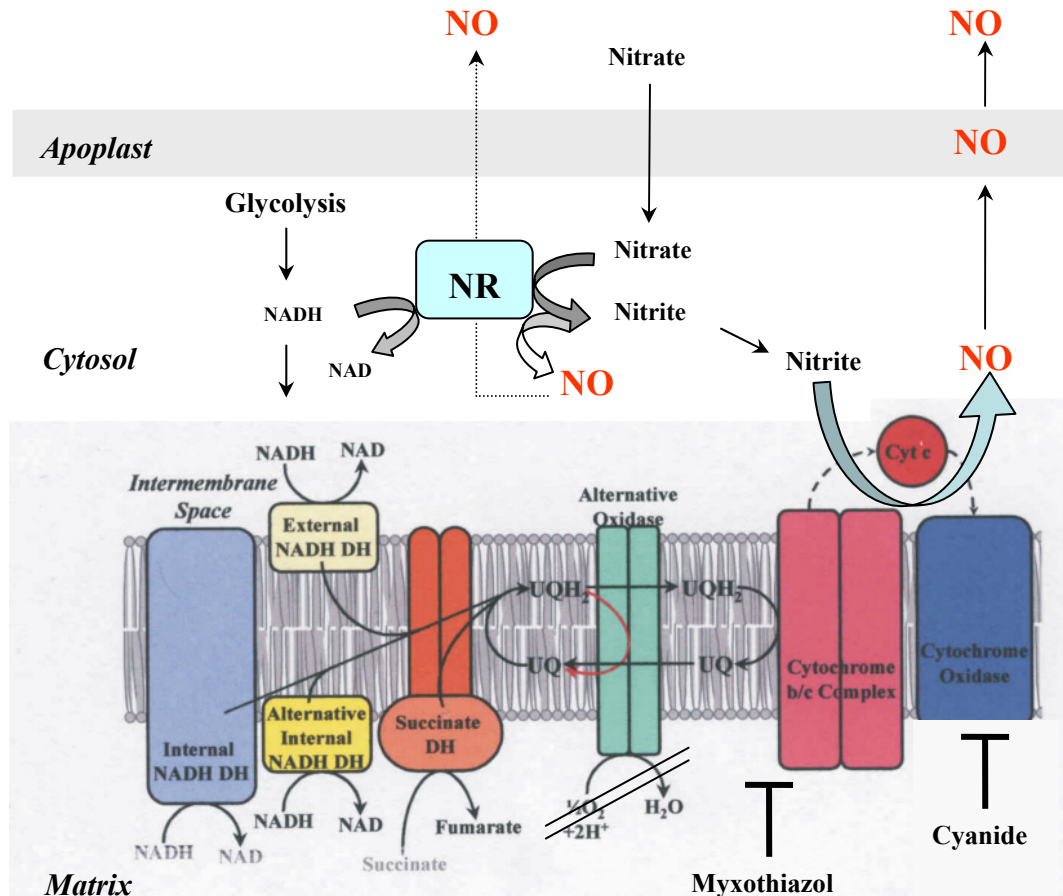
While the ability of NR to produce NO from nitrite and NADH was beyond any doubt, literature data have suggested several other potential sources for NO. In our hands, NR-free plants did not emit any NO even under anoxia. However, with nitrite added, anoxic cell suspensions (NR-free because grown on ammonium) gave normal rates of NO emission. Like NR-free leaves, these cells were not able to emit NO under normal conditions. Clearly, cells had an NR-independent, but also nitrite-dependent (and probably NADH)-dependent NO production. Several studies in animal physiology reported that xanthine dehydrogenase (XDH), an ubiquitous MoCo-enzyme, could reduce nitrite to NO under anoxia in the presence of NADH (Millar *et al.*, 1998, Godber *et al.*, 2000; Li *et al.*, 2001). Our results do not confirm an involvement of XDH for two major reasons: Tungstate-grown cell suspensions lacking the functional MoCo were also able to produce NO when supported with nitrite. Further, recombinant purified XDH produced absolutely no NO from nitrite plus NADH (unpublished, cooperation with R. Mendel, Braunschweig, Germany).

One other possible source for nitrite-dependent NO is a nitrite:NO oxido-reductase localized in the plasma-membrane (PM), detected by Stöhr *et al.* (2001). However, this activity was so far ascribed to roots only. Nothing is known about its presence in cell suspensions.

Using EPR, Kozlov *et al.* (1999) demonstrated that mammalian mitochondria had the ability to reduce nitrite to NO under anaerobic conditions at the same site involved in the one electron reduction of O<sub>2</sub>. Evidence on the involvement of the redox couple between ubiquinol and complex III came from the total inhibition of nitrite reduction by myxothiazol. In our tungstate-grown cell suspensions, anaerobic, nitrite-dependent NO emission was inhibited by cyanide, indicating a possible involvement of a heme-protein, eventually the cytochrome-bc1-reductase (complex III). Preliminary experiments with NR-free *Chlorella* confirmed the existence of a myxothiazol-sensitive anoxic NO emission from nitrite (R. Tischner and W.M. Kaiser, unpublished results), and SHAM, an inhibitor of the AOX pathway, stimulated NO emission, which may indicate that blockage of the AOX pathway would divert electrons into the cytochrome-bc-reductase and into the cytochrome oxidase pathway for reduction of nitrite to NO. According to these preliminary

data (not shown here), a model has been developed showing the hypothetical model of mitochondrial NO production (Figure 66).

It is important to note, however, that the above-postulated mitochondrial NO production still requires nitrite, which- at least in higher plants not exposed to environmental NO<sub>x</sub> - is exclusively produced by NR.



**Figure 66:** A hypothetical interaction of the respiratory chain of plant mitochondria (under anaerobic conditions) with nitrite-dependent NO production.

Under anoxia, nitrite (from NR-induced cells) in contact with respiring mitochondrial could accept reducing equivalents from the ubiquinone cycle to the b/c complex of the respiratory chain or to the cytochrome oxidase and finally would produce NO. The scheme for mitochondrial electron transport was taken from <http://www.public.iastate.edu/~botany/bot513/2002/lectures/LCT11.pdf>.

## 4 NO in the cryptogein-induced HR: NO scavengers

Cryptogein has been shown to provoke a rapid NO burst from epidermal peels (Foissner *et al.*, 2000) which was prevented by c-PTIO. In our hands, the NO scavenger PTIO (as well as c-PTIO and tma-PTIO) very efficiently prevented cell death in leaves or cell suspensions, though only at very high concentrations. Thus, our findings were in line with previous reports (Foissner *et al.*, 2000; Delledonne *et al.*, 1998; Durner *et al.*, 1998; Clarke *et al.*, 2000).

If NO production in response to cryptogein would be restricted to an early phase during the HR, as suggested by Foissner *et al.* (2000), addition of a scavenger after that phase should have no effect on the HR. At least with cell suspensions, this was not the case: At any time after cryptogein addition, c-PTIO addition kept the percentage of dead cells on the status quo and prevented any further increase in cell death. This is in clear contrast to the idea of a homogeneous synchronized NO burst as a trigger for cell death. Rather, our cell suspensions were responding to cryptogein in a non-synchronous manner, since the percentage of dead cells increased almost linearly with time, following cryptogein addition. This would also suggest a “multiple switch model” (Figure 67), where each single cell would be triggered to cell death at individual times. The observed time course of the PTIO effect would be consistent with that model.

CHX inhibited the cryptogein-induced cell death in tobacco leaves, indicating that the HR requires *de novo* protein synthesis. Hypersensitive cell death caused by elicitors is thought to be a kind of PCD, and PCD is dependent on both RNA and protein synthesis. Similar results were reported on harpin effects (Desikan *et al.*, 1998; Andi *et al.*, 2001). The induced genes required for the expression of the HR would certainly include PR proteins, and our data showed a strong induction of PR-1 by cryptogein.

## 5 NO-emission during the HR: chemiluminescence

The above measurements have shown that ozone-mediated chemiluminescence NO-detection is basically suited for real-time quantification NO production in plants, organs, cells or solutions. In studies on plant-pathogen interactions, the method has been rarely



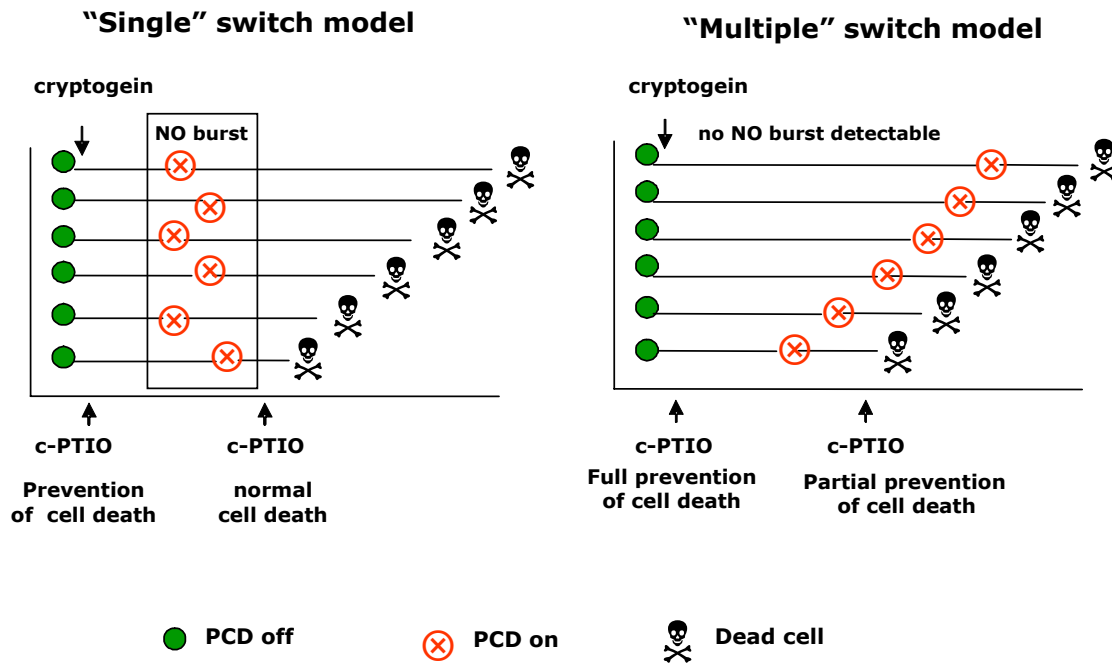
used. While the response of leaves to the NO scavenger PTIO suggests that NO plays an important role in the HR, chemiluminescence measurements failed to detect any additional NO production after cryptogein infiltration into leaves.

As already mentioned, using DAF-2DA fluorescence, Foissner *et al.* (2000) could detect a rapid NO burst after six minutes into tobacco epidermal peels treated with cryptogein. As the highly fluorescent reaction product of DAF with NO (or rather the oxidation product: NO<sup>+</sup>), DAF 2T, is rather stable and accumulates during continuous NO production, this lack of sensitivity is counterbalanced by the accumulative signal. The detection limit by DAF for NO is 5 nM (Kojima *et al.*, 1998). Under our conditions, when normal, NR-dependent NO emission from illuminated leaves in air reached a steady state of ~ 0.20 nmol gFW<sup>-1</sup> h<sup>-1</sup>, the NO concentration in the gas phase was about 0.17 ppb, indicating a minimum NO concentration in the water phase of 0.32 pM, if we consider that the diffusion of NO out of the aqueous phase (apoplast of leaves) should be equal to the NO net production rate, and the NO concentration in the aqueous phase should be constant. The Henry coefficient for NO at atmospheric pressure is 1.9 mM. Under these conditions, the equilibrium concentration of NO in the gas phase above a 5 nM solution would be 2.5 ppm, and thus up to 15000 fold higher than normally found by chemiluminescence in the gas flow passing over an illuminated leaf from nitrate-grown tobacco. In the other hand, DAF-fluorescence is strongly pH-dependent and also depends on free Ca<sup>2+</sup>. Indeed, cryptogein has been shown to induce a rapid cytosolic acidification of about 0.4 pH (Pugin *et al.*, 1997), together with an alkalinisation of the apoplast. Acidification, however, should decrease DAF-fluorescence (Kojima *et al.*, 1998), whereas pathogens or elicitors usually increase DAF-fluorescence. Nevertheless, it seems possible that fluorescence changes of DAF may have other origins than just NO. Indeed, the specificity of DAF-2 has been rigorously tested by Jourdeuil (2002) who concluded that the products derived from the autoxidation of NO are essential for the nitrosation of DAF-2 to the fluorescent form DAF-2T, although the probe can, under certain conditions, react both with N<sub>2</sub>O<sub>3</sub> (from the oxidation of NO) and with the NO formed from the dissociation of ONOO<sup>-</sup>. The intracellular oxidation of DAF-2DA may result in increased NO-dependent fluorescence that may be mistaken as an increase in NO-production. This may explain the 5 nM detection limit for NO previously reported for the DAF-2 assay in cells (Kojima *et al.*, 1998), which is incompatible with the extremely slow autoxidation of NO at nanomolar

concentrations. Previous studies (Broillet *et al.*, 2001, Suzuki *et al.*, 2002) recommended greater caution for the interpretation of DAF-2 fluorometric assays.

While cryptogein induced no additional NO emission from tobacco leaves, induction of NO emission was observed with tobacco cell suspensions, which produced NO at a low rate of  $\sim 0.7 \text{ nmol gFW}^{-1} \text{ h}^{-1}$  after 3-6 h elicitation (Figure 47), which corresponds to a minimum NO concentration in the water phase of 0.28 pM (concentration comparable with illuminated tobacco leaves). The relatively late starting point for NO emission, and the fact that the NO emission rate remained constant thereafter, makes it difficult to consider NO as an early signalling component triggering the HR. These kinetics of NO generation by cryptogein-treated tobacco cells differed from those reported for soybean cells challenged by an avirulent pathogen (Delledonne *et al.*, 1998). Using the hemoglobin method, these authors found a biphasic NO-production, where the second phase was characterized by transient NO production at 6 h. The NO concentration in the solution required to trigger the HR was 1 to 2  $\mu\text{M}$ , which would correspond to a gas phase equilibrium concentration of 1000 ppm, or  $7 \times 10^6$  higher than the above measured concentration by chemiluminescence. Obviously, the NO concentrations required to provoke the HR as given by Delledonne *et al.* (1998) are drastically overestimated. Using the same method (hemoglobin assay), Clarke *et al.* (2000) found a rapid and sustained elevation of NO production with *Arabidopsis* cells inoculated with an avirulent pathogen, which started within 30 min after bacterial challenge and was maintained at  $1\text{-}1.5 \text{ nmol g wetW}^{-1} \text{ min}^{-1}$  for a 6 h period. This NO production rate is approximately 150 fold higher than our rate.

We have discussed above that our cell suspensions responded non-synchronously to cryptogein, as the percentage of dead cell increased linearly with time. The kinetic of NO production in the Delledonne's data showed a distinct maximum around 6 h. Such a 'NO burst' from a cell suspension inevitably requires a synchronous response of the cells. Also, the sharp and rapid NO burst detected only six minutes after cryptogein-treatment of epidermal tobacco cells would require a perfect synchronisation of leaf epidermal cells (Foissner *et al.*, 2001). In contrast to the suggested 'multiple switch' in our model, a synchronized induction of cell death would follow a 'single switch' model (Figure 67).



**Figure 67:** Hypothetical “single” and “multiple” switch models in cryptogein treated cells.

In the single switch model, adding c-PTIO to cryptogein-treated synchronous cells before the NO burst should prevent cell death addition, and after this NO burst, should have no effect on cell death. In the case of a multiple switch model, individual NO burst would be too small to be detected (cells non-synchronous), and adding c-PTIO at any time before the induction of PCD would prevent cell death thereafter. The “multiple” switch model is consistent with our own observations, but is in marked contrast to the literature (see text).

It has been suggested that elicitors or pathogens induce an oxidative burst (Bottin *et al.*, 1994; Viard *et al.*, 1994; Tavernier *et al.*, 1995; Rustérucchi *et al.*, 1996; Simon-Plas *et al.*, 1997) which closely matched NO production. Such a parallel production of ROS and NO may cause problems. For example, hemoglobin can react with both compounds, and consequently, the detection of NO only could be altered. Contrary to the hemoglobin method, chemiluminescence specifically detects NO released from the plant cells. However, NO formed upon cryptogein treatment may be trapped by parallel ROS production, such that no additional NO would escape into the gas phase, at least from leaves (Figure 47). This idea is supported by the observation that quenching of added NO was higher in cryptogein-treated cell suspensions as compared to the controls (Figure 65), and that *in vitro*-NO emission from solutions of purified NR was very efficiently quenched

by addition of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (Figure 19). Indeed, a careful estimation of  $\text{H}_2\text{O}_2$  concentrations in barley leaves gave values of 40-120  $\text{nmol g}^{-1}$  FW, well in the above range (Veljovic-Jovanovic *et al.*, 2002). There is also no doubt that, due to the reactivity of NO, much of the NO produced inside cells would react with cellular constituents (Figure 11), such that only a small and eventually variable part would escape into the gas phase to be detected by chemiluminescence. However, this problem is not restricted to gas phase chemiluminescence detection. Any indicator reaction (like the DAF-2DA-fluorescence, which is less specific) has to compete with other reactions of NO inside cells. Accordingly, any change in the apparent NO concentration may either be due to changes in NO production or to changes in NO quenching, except if the affinity of the indicator would be much higher than the affinity of the competing reactions, which is not known with certainty. Quenching of NO may be different for different tissues or plant organs, and that may be one reason why we could detect cryptogein-induced NO emission from cell suspensions, but not from leaves. Further, we can not completely exclude that in tobacco leaves the “signalling NO” is produced only locally, which may cause only very small changes in total NO emission from the leaves. However, in that case the serious problem would be how the cells can respond to a signal that hardly exceeds the rather high background level of NO that was found in nitrate reducing cells, or that may be caused by high atmospheric NO<sub>x</sub> levels in polluted air, or in unstirred air layers above soil with NO producing microorganisms.

## 6 NO-source(s) during the HR?

In most of the available literature on NO production during the HR, NOS was suggested as the source for NO, mainly based on the fact that NOS-inhibitors prevented cell death. In our experiments, cryptogein-induced NO-emission from cell suspensions was completely insensitive to L-NAME (2 mM), as was cell death. Lesion developments after cryptogein-infiltration in to leaves were not or only weakly responsive even to high concentrations (50 mM) of NOS-inhibitors. Our data were supported by a recent report by Clarke *et al.* (2000) that treatment of *Arabidopsis* cultures with NOS inhibitors (L-NNA or L-NAME) did not reduce NO-generation following bacterial challenge, in contrast to the situation with soybean cultures, in which NOS inhibitors were reported to reduce NO generation (Delledonne *et al.*, 1998). Durner’s group has shown that the burst of DAF-2DA

fluorescence detected after few minutes was only partially inhibited (50 %) by a NOS-inhibitor (Foissner *et al.*, 2000). The specificity of NOS inhibitors may differ with the plant NOS isoforms (Durner *et al.*, 1998).

We detected only very low NOS-like activities in leaf extracts, which did not respond (increase) to elicitor treatment (Figure 54), as should be expected if cryptogein would have induced an iNOS (Chandok *et al.*, 2003), or if it had activated a constitutive enzyme. Also, the NOS-like activity that we measured did not respond to NOS inhibitors *in vitro*. We therefore assume that these low rates of L-arginine consumption were not due to a mammalian-type NOS, but to some unidentified reaction. Following TMV infection, Chandok *et al.* (2003) have shown that NOS activity from tobacco leaf extract, determined by the oxy-hemoglobin assay, was sensitive to L-NMMA. 90 % of this activity could be suppressed by 1  $\mu$ M L-NMMA, which is acting as a competitive substrate analog. Considering that L-arginine represents one of the most abundant amino acids in plants, being often in the millimolar range, the sensitive inhibition by such low L-NMMA concentrations is somewhat surprising. In the other hand, in response to TMV inoculation, NOS activity in tobacco leaves increased continuously during 22 h post inoculation (Chandok *et al.*, 2003). Clearly, TMV infection led to a continuously increasing NOS activity, which is inconsistent with the idea of a “NO-signalling burst”. Indeed, the linear time course of cryptogein-induced cell death (Figure 40) and the induction of the NOS-like activity after TMV infection (Chandok *et al.*, 2003) appear surprisingly similar.

The fact that NR could produce NO under normal conditions and that NOS seemed not involved in PCD, 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 cryptogein-induced event was a nitrate efflux (Wendehenne *et al.*, 2002), and that NR was induced by pathogen signals (Yamamoto *et al.*, 2003). However, in our experiments, cryptogein- induced lesion formation in tobacco leaves, or cell death of cell suspensions, was absolutely independent of NR. Strange enough, in the case of cell suspensions, NO emission could be detected few hours after cryptogein addition, but only in nitrate grown cells, not in NR-free ammonium cells. Thus, the induction of NO formation in these cell suspensions was not related to NO emission.

While many of our results actually question the often proposed role of NO in the induction of cell death, the efficient prevention of cell death by c-PTIO requires additional

assumptions. c-PTIO reacts with NO to produce c-PTI and this latter does not scavenge NO (Figure 41). Surprisingly c-PTI, like c-PTIO prevented lesion formation after cryptogein treatment (Figure 42). Accordingly, the inhibition of the HR by c-PTIO is no real proof for the participation of NO, and the action profile of c-PTIO may be more complex than hitherto assumed. Also, the specificity of c-PTIO as NO-scavenger was questioned (Pfeiffer *et al.*, 1997), and strong reductants (*e.g.* ascorbic acid) generate c-PTI from c-PTIO in the absence of NO (Az-ma *et al.*, 1994).

## 7 NO and ROS, independent pathways to induce cell death?

It is still a controversial question whether NO alone is sufficient to induce cell death, or whether a concerted action of ROS and NO are required (Delledonne *et al.* 2001). In *Arabidopsis* cell suspensions, NO donors alone were found to induce cell death when applied at concentrations that released NO at amounts similar to those generated by cells challenged by avirulent bacteria (Delledonne *et al.*, 1998; Clarke *et al.*, 2000; Delledonne *et al.*, 2002). However, results obtained with NO-donors, and particularly SNP, should be considered with caution (Wink *et al.*, 1996). Decomposition of SNP is affected by several factors (such as transition metals, reducing agents, light radiation or enzymatic metabolism) (Feelisch, 1998), and NO is probably released in the form of  $\text{NO}^+$ , which is much more reactive than NO itself and thus may have effects different from those of NO (Stamler *et al.*, 1992).

In tobacco leaves of clone 271, which have a continuous and drastic overproduction of NO especially in the light, cell death was obviously not induced, although cells became chlorotic after prolonged growth on nitrate. NO overproduction also had no visible effect on cryptogein-induced lesion development, which contrasts the suggestions by Delledonne *et al.*, (2001). It is interesting in that context that in leaves of these transformants, a high level of nitrotyrosin was found, probably indicating  $\text{ONOO}^-$  formation (Morot-Gaudry-Talarmain *et al.*, 2002).

On the other hand, the oxidative burst is thought to trigger and orchestrate the various active defense responses (Levine *et al.*, 1994; Chamnongpol *et al.*, 1998; Desikan *et al.*, 1998). In challenged soybean cell suspension cultures, ROS were reported to act as a local trigger of programmed death and as a diffusible signal for the induction of genes encoding

cellular protectants in adjacent cells (Levine *et al.*, 1994). Our experiments with DPI and with the antioxidant enzymes SOD and catalase suggest that ROS are not required for the cryptogein-induced hypersensitive cell death in tobacco cells (Figure 64). Similar conclusions were drawn by Dorey *et al.* (1999) and by Binet *et al.* (2001). Devlin and Gustine (1992) reported also that incubation of bacteria with SOD and catalase before infiltration into tobacco leaves did not interfere with development of the HR. The hypersensitive cell death induced in tobacco cell suspensions by two proteinaceous HR elicitors, INF1 elicitor from *P. infestans* (Sasabe *et al.*, 2000) and harpin from *Erwinia amylovora* (Xie and Chen, 2000) was not prevented by ROS inhibitors or scavengers. In contrast, Rustérucci *et al.* (1996) reported a correlation between a cryptogein-induced oxidative burst in tobacco cell cultures and the capacity of cryptogein to induce tissue necrosis on tobacco leaves. The study of Levine *et al.* (1994) indicated that H<sub>2</sub>O<sub>2</sub> itself might trigger cell death. However, the relatively low concentration of ROS produced during an incompatible plant/bacteria interaction was not sufficient to trigger the HR (Baker *et al.*, 1995; Glazener *et al.*, 1996).

If NO and ROS would be produced in parallel in such a way that NO emission would be completely prevented, one should at least expect some weakening or delay in lesion development by infiltration with catalase and/or or SOD, and some increase in NO emission due to less quenching. This was not observed, contrary to the data from Mayama and coworkers (2004) who found that SOD treatment increased NO accumulation during the defense response of oat. Some reports claim that very high concentrations of catalase are required to prevent HR in leaves (Doke *et al.*, 1988; Masuta *et al.*, 1991; Levine *et al.*, 1994; Desikan *et al.*, 1996; Willekens *et al.*, 1997) but it is unknown at present whether very high catalase concentrations may cause artefacts *e.g.* by interacting directly with NO and then abolishing the signal transduction.

Synergistic effects of exogenous NO and ROS in triggering the death of plant cells open the possibility that ONOO<sup>-</sup> toxicity may be responsible for cell death in plant-pathogen interactions (Bolwell, 1999; Durner and Klessig, 1999). Using direct application of ONOO<sup>-</sup> (SNP with O<sub>2</sub><sup>-</sup>-generating system hypoxanthine/XO), Alamillo and Garcia-Olmedo (2001) showed that ONOO<sup>-</sup> did produce necrotic lesions in plants and that urate, an ONOO<sup>-</sup> scavenger, was able to attenuate or to protect against the phytotoxic effect of ONOO<sup>-</sup>, proving that ONOO<sup>-</sup> plays a significant role in the responses of plants to *Pseudomonas*

*syringae*. In the other hand, Delledonne *et al.* (2001) showed that the addition of ONOO<sup>-</sup> to soybean cell suspensions did not affect cell viability. Clarke *et al.* (2000) showed that the programmed cell death was independent of the formation and the concentration of ONOO<sup>-</sup>, which suggested a separate regulatory role of NO.

## 8 Open questions

While it is clear that NR is a major source for NO in plants, the contribution of at least one other system, eventually located in the mitochondria, seems obvious. Its identification and characterization requires further investigations. The role of NO in the HR is still questionable, be it as a signal or as a toxic substrate. If we accept the existence of NOS in plants, it is not clear how different NO producing systems interact with each other. The integration of our findings into the established picture of NO signalling is difficult, if not impossible. Transgenic *Arabidopsis* plants, expressing a bacterial NOS, are presently developed in our lab, and these plants may help for a better understanding of the role of NO as a universal signal regulating plant growth and plant/pathogen interactions.



## D Material and Methods

### 1 Plant material and growth conditions

#### 1.1 Tobacco plant growth conditions

Experiments were conducted with *Nicotiana tabacum* cv. *Xanthi* (NN) and *Nicotiana tabacum* cv. *Gatersleben*. A nitrite-reductase antisense-transformant (clone 271) was obtained from INRA Versailles, France. A nitrate reductase (NR)-deficient *nia30* double mutant was obtained from R. Mendel, Braunschweig, Germany. These two mutants are derived from the WT *Nicotiana tabacum* cv. *Gatersleben*.

Seeds were germinated on vermiculite in a day-night regime of 14/10h, 24/20°C, a relative humidity of 80 % and 350-400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. After three weeks, the plants were transferred to hydroponic culture for additional four to six weeks. Plastic pots, each containing 1.8 L nutrient solution, were kept in a growth chamber with artificial illumination (HQI 400w, Schreder, Winterbach, Germany) at a photon flux density (PAR) of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and with 16 h daily light periods. The day/night temperature regime of the chamber was 24°C/20°C respectively.

According to the different requirements, the following nutrient solutions were used:

#### ■ Nitrate nutrient solution (pH 6.3)

KNO <sub>3</sub>	10 mM
CaCl <sub>2</sub>	1 mM
MgSO <sub>4</sub>	1 mM
NaFe-EDTA	25 $\mu\text{M}$
K <sub>2</sub> HPO <sub>4</sub>	0.5 mM
KH <sub>2</sub> PO <sub>4</sub>	1 mM
<i>Micro-elements</i>	
H <sub>3</sub> BO <sub>3</sub>	15 $\mu\text{M}$
MnCl <sub>2</sub> , 4 H <sub>2</sub> O	3 $\mu\text{M}$
ZnSO <sub>4</sub> , 7 H <sub>2</sub> O	0.25 $\mu\text{M}$
CuSO <sub>4</sub> , 5 H <sub>2</sub> O	0.1 $\mu\text{M}$
Na <sub>2</sub> MoO <sub>4</sub>	0.04 $\mu\text{M}$

■ Ammonium nutrient solution (pH 6.2)

NH <sub>4</sub> Cl	3 mM
CaCl <sub>2</sub>	1 mM
MgSO <sub>4</sub>	2 mM
NaFe-EDTA	25 μM
K <sub>2</sub> HPO <sub>4</sub>	0.5 mM
KH <sub>2</sub> PO <sub>4</sub>	1 mM
<i>Micro-elements</i>	
H <sub>3</sub> BO <sub>3</sub>	15 μM
MnCl <sub>2</sub> , 4H <sub>2</sub> O	3 μM
ZnSO <sub>4</sub> , 7H <sub>2</sub> O	0.25 μM
CuSO <sub>4</sub> , 5H <sub>2</sub> O	0.1 μM
Na <sub>2</sub> MoO <sub>4</sub>	0.04 μM

As growth of clone 271 and *nia30* was slower than that of WT (*Nicotiana tabacum* cv. Gatersleben), seeds of clone 271 and *nia30* were sown in vermiculite up to two months earlier than WT. When the growth state of these plants was comparable with WT, the plants were also transferred to hydroponic culture.

NiR-deficient transformants (clone 271) were pre-grown on ammonium-nutrient solution (as above), and one week before harvest, plants were transferred into the above nitrate nutrient solution (if not mentioned otherwise).

Nitrate reductase (NR)-deficient *nia30* mutant plants were grown on ammonium nutrient solution with 2 mM K<sub>2</sub>HPO<sub>4</sub> instead of 0.5 mM in order to have a higher starting pH (6.2). Before using these plants for experiments, they were transferred for one week to a nutrient solution containing 3 mM NH<sub>4</sub>NO<sub>3</sub> and 1 mM NH<sub>4</sub>Cl as nitrogen source.

All nutrient solutions were changed three times a week and again on the day before experiments. For the tungstate treatment, four to eight week old WT plants were fed daily for five days with full strength nitrate nutrient solution containing 500 μM sodium tungstate instead of molybdate (to inhibit NRA) and 1 mM NH<sub>4</sub>Cl (to avoid N starvation) in addition. The solution was changed every day during five days of treatment before experiments.

## 1.2 *Nicotiana tabacum* cell suspension cultures

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.

### *Linsmaier & Skoog medium (LS medium)*

#### Macro-elements

$\text{NH}_4\text{NO}_3$	20.61 mM
$\text{KNO}_3$	18.79 mM
$\text{MgSO}_4, 7\text{H}_2\text{O}$	1.50 mM
$\text{KH}_2\text{PO}_4$	1.25 mM
$\text{CaCl}_2, 2\text{H}_2\text{O}$	2.99 mM

#### Micro-elements

$\text{Na}_2\text{EDTA}, 2\text{H}_2\text{O}$	0.1 mM
$\text{FeSO}_4, 7\text{H}_2\text{O}$	0.1 mM
$\text{H}_3\text{BO}_3$	0.1 mM
$\text{MnSO}_4, \text{H}_2\text{O}$	0.1 mM
$\text{ZnSO}_4, 7\text{H}_2\text{O}$	29.91 $\mu\text{M}$
KI	5.0 $\mu\text{M}$
$\text{Na}_2\text{MoO}_4, 2\text{H}_2\text{O}$	1.03 $\mu\text{M}$
$\text{CuSO}_4, 5\text{H}_2\text{O}$	0.10 $\mu\text{M}$
$\text{CoCl}_2, 6\text{H}_2\text{O}$	0.11 $\mu\text{M}$

#### Vitamins

Myo-inositol	100.0 $\text{mg L}^{-1}$
Thiamine HCL	0.40 $\text{mg L}^{-1}$

#### Hormones

Auxin	0.22 $\text{mg L}^{-1}$
Cytokinin	0.18 $\text{mg L}^{-1}$

#### Sucrose

30  $\text{g L}^{-1}$

Ammonium cell suspension cultures totally devoid of nitrate were grown on LS medium with small modifications: 1.5 mM NH<sub>4</sub>Cl instead of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub>, 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 Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O was replaced by tungstate (150 μM).

## 2 Experimental treatments

### 2.1 Cryptogein treatment

Cryptogein, the fungal elicitor from *Phytophthora cryptogea*, was purified according to Ricci *et al.* (1989) and was kindly provided by Dr. H. Keller (INRA, Unité Santé Végétale et Environnement, Antibes, France). The lyophilized cryptogein (2.6 mg) was resuspended in water (260 mL) and immediately dispatched into 1 mL aliquots and kept at -20°C.

#### ■ on tobacco leaves

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).

#### ■ on tobacco cell suspensions

Cells from cultures in exponential growth phase (subcultured after three days) were washed with LS medium containing only the macro-elements and sucrose. The cells were adjusted to a cell density of 0.1 gFW mL<sup>-1</sup>. Before any treatment, cells were allowed to adjust to the new conditions for a period of 30 min-1 h.

## 2.2 Chemical treatments

Some enzymes or chemicals were injected in the nutrient solution of detached tobacco leaves (four to eight week), hand-infiltrated into tobacco leaves or injected in cell suspensions with or without elicitors and the effects scored after an appropriate time-treatment. When using several products, they were mixed at the adequate concentration prior to infiltration or to addition. Diluted samples were prepared just before use and were protected from light, heat and air during a short time (For a list of enzymes and chemicals see section 8).

## 3 Preparation of NO solutions

■ NO saturated water was prepared by gently bubbling NO gas (100 ppm in nitrogen; Messer, Griesheim, Darmstadt, Germany) for 15 min at 24°C, into a glass vial filled with HEPES (100 mM pH 6.5) or MES (50 mM, pH 5.5) buffer. The concentration of a gas dissolved in a liquid can be calculated by Henry's law. The solubility of a gas is proportional to the pressure of the gas over the solution. The concentration of NO in a saturated solution was taken as 1.9 mM. Then, at 24°C and at atmospheric conditions, the NO equilibrium concentration in the 1 mL solution flushed with 100 ppm NO is 190 pmol.

■ NO was also generated using the spontaneous decomposition of the NO-donor (sodium nitroprusside; SNP, Merck, Darmstadt, Germany). The NO-donor solution was freshly prepared prior to experiments as a 50 mM stock solution and kept in dark and stored on ice until used.

## 4 Cell death assay

Evan's blue is a non permeating dye that can enter the cells only through damaged/ruptured plasma membranes and stain the contents of dead cells. For the Evan's blue assay, tobacco cell suspensions were incubated for 15 min at room temperature with 0.05 % (in water) Evan's blue solution (Fluka, Taufkirchen, Germany). Subsequently, cells

were placed in a spin column and pelleted by centrifugation at 100 rpm for 1 min and washed 5-6 times with distilled water until no more dye was eluted from the cells. Dye firmly bound to dead cells was solubilized in 50 % methanol with 1 % SDS for 30 min at 55°C, and quantified (after removal of cells residues by centrifugation) by measuring the absorbance of the supernatant at 600 nm (Levine *et al.*, 1994). 100 % cell death was obtained by a freeze/thaw cycle.

## 5 *In vitro* enzymatic activities

### 5.1 Nitrate reductase activity and nitrite content

■ Following different treatments, leaf discs or leaves cut into halves along the midrib were taken and were 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 µM FAD, 10 µM molybdate, 15 mM MgCl<sub>2</sub>, 2 mM pepabloc, 50 µM leupeptin, 50 µM cantharidine, 0.5 % PVP, 0.5 % BSA and 0.3 % of triton) was added to one gFW. Cantharidine (a PP2A inhibitor) was added 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 µL extract, 4°C) equilibrated with the extraction buffer without the protease-inhibitors. With aliquots of the supernatant the following assays were carried out:

• **Determination of NRA actual (+ Mg<sup>2+</sup>):**

200µL leaf extract was added to 800 µL reaction mixture (100 mM HEPES-KOH pH 7.6, 1 mM DTT, 10 µM FAD, 10 µM molybdate, 15 mM MgCl<sub>2</sub>, 5 mM KNO<sub>3</sub> and 0.2 mM NADH). The reaction was carried out as below.

• **Determination of NRA maximal (+ EDTA):**

200µL leaf extract as above, but containing in addition 20 mM EDTA and 5 mM AMP (final concentrations) were preincubated at 24°C. After 13 min, buffer (100 mM HEPES-KOH pH 7.6, 1 mM DTT, 10 µM FAD, 10 µM molybdate and 15 mM EDTA) was added

to a final volume of 1 ml. After 2 min (total preincubation time: 15 min), the reaction was started by adding 5 mM KNO<sub>3</sub> and 0.2 mM NADH (final concentrations).

After 5 min (24°C), the reaction was stopped by adding 125 µL zinc acetate (0.5 M). Following centrifugation (16000g, 5 min), the supernatant was treated with 10 µM phenazine methosulphate (PMS) to oxidize un-reacted NADH. The colorimetric determination of formed nitrite was carried out as described previously by Hageman and Reed (1980).

■ Aliquots of the cell culture were gently vacuum-filtrated through cold cellulose filters (5 cm diameter) in order to remove the medium. The cells were scraped off the filters either directly into a mortar or first on a piece of aluminium foil. In the both cases, they were immediately frozen in liquid nitrogen and either extracted directly or kept at -80°C until further use. The NRA was afterwards measured in the same way as with leaf extracts.

The nitrite content was usually determined from cells plus medium, and was expressed in nmol mL<sup>-1</sup>.

## **5.2 Nitric oxide synthase activity**

NOS activity was measured by an arginine / citrulline assay according to the method of Ninnemann and Maier (1996), with slight modifications. Leaf material was ground with liquid nitrogen in a mortar and 1 mL extraction buffer (50 mM HEPES-KOH, pH 7.4, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10 µM leupeptine, 1 µM pepstatine) was added per 1 gFW. After continuous grinding until thawing the suspension was centrifuged (14500 g, 10 min, 4°C). In order to remove endogenous arginine the supernatant was desalted on Sephadex G-25 spin column equilibrated with the extraction buffer. 40 µL leaf extract was added to 60 µL reaction mixture (50 mM HEPES-KOH, pH 7.4, 10 µg calmodulin, 1 mM CaCl<sub>2</sub>, 0.5 mM EDTA, 5 µM FAD, 5 µM FMN, 10 µM tetrahydrobiopterin, 0.3 mM DTT, 1 mM NADPH, 10 µM L-arginine (including 3.7 kBq L-[2,3,4,5-<sup>3</sup>H] arginine, Amersham Pharmacia Biotech, Braunschweig, Germany). After incubation at 25°C for 30 min, the reaction was stopped with pre-cooled 1.4 mL 20 mM HEPES-KOH, pH 5.5 containing 2 mM EDTA. Unreacted labeled arginine was removed by a batch procedure with Dowex

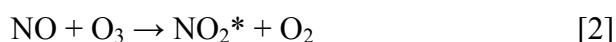
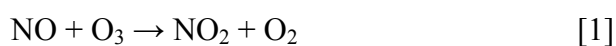
50WX-8 (H<sup>+</sup> form, 200 – 400 μm mesh, Fluka). Before use, the resin was washed with 0.1 N NaOH (30-times the resin volume), pelleted and washed three times in 4-times the resin volume of a solution containing 20 mM HEPES-KOH, pH 5.5 and 2 mM EDTA and adjusted pH to 5.5. Five hundred mL of above reaction mixture was added to 500 μL of the resin and mixed for 15 min with a rotary shaker. Following centrifugation (1500 g, 5 min), radioactivity in 400 μL of the supernatant was quantified by a liquid scintillation counting. Radioactivity of a blank without leaf extract was subtracted from each sample value. Protein concentrations were determined with protein assay kit (Bio-Rad Laboratories Inc. Munich, Germany) using bovine serum albumin as a standard.

## 6 Gas phase NO measurements

### 6.1 General description and basics of the chemiluminescence detection

#### 6.1.1 Measurement principle

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<sub>3</sub>). NO is measured directly, NO<sub>2</sub> indirectly. The reactions between NO and ozone can be described by the following formulae:



NO<sub>2</sub>\*: the excited nitrogen dioxide molecule

M: deactivating colliding partners (N<sub>2</sub>, O<sub>2</sub>, H<sub>2</sub>O)

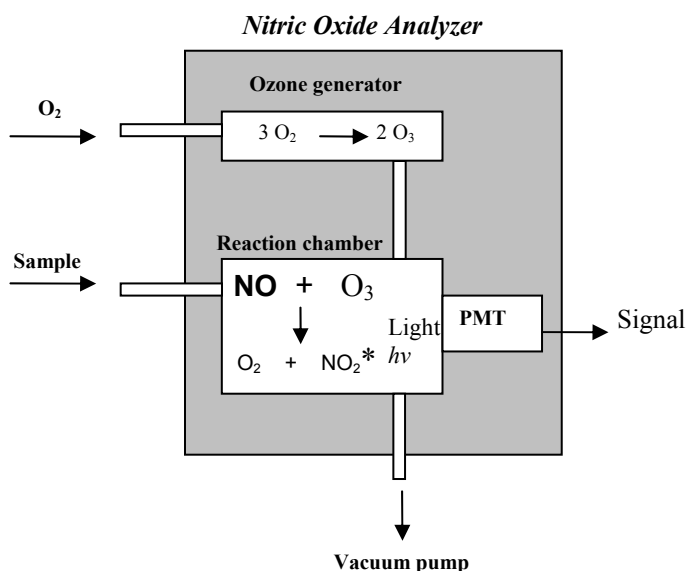
The method is based on a first order chemical reaction between NO and an excess amount of ozone (O<sub>3</sub>) [1]. A significant fraction of nitrogen dioxide (NO<sub>2</sub>) produced in the reaction is in an excited state NO<sub>2</sub>\* [2]. The spontaneous deactivation of NO<sub>2</sub> occurs with emission of light [3] where each molecule of NO<sub>2</sub>\* emits one photon. By far the larger fraction of NO<sub>2</sub>\* loses its excitation energy without light emission by colliding with other molecules



(M) [4]. The light intensity generated from the chemiluminescence reactions [2] [3] is proportional to the mixing ratio of NO. A red sensitive photomultiplier tube (PMT) is used to convert the light energy emitted from [3] into electrical impulses.

### 6.1.2 Description of the analyzer CLD 770 AL ppt

The figure 68 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. 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<sub>3</sub> 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.



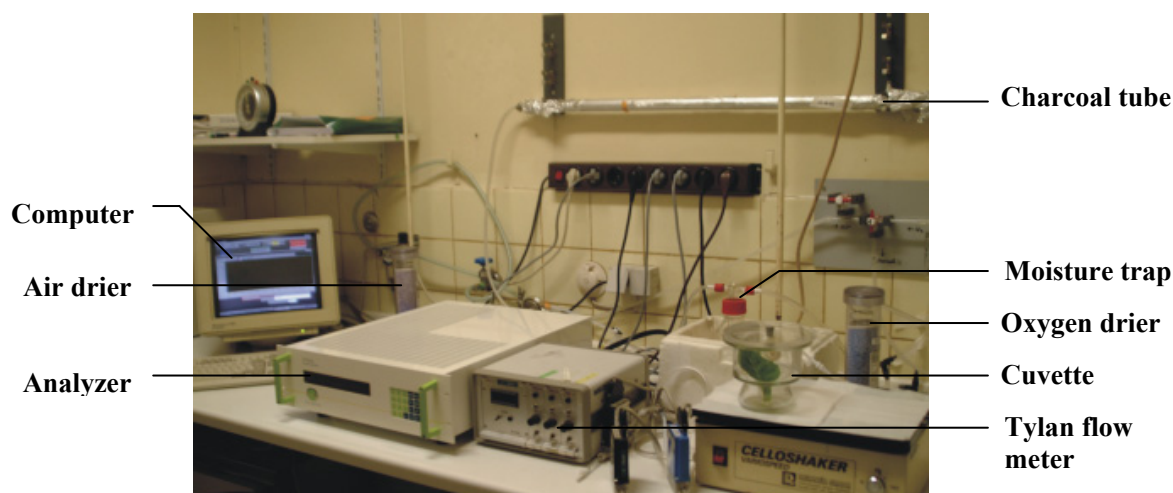
**Figure 68:** Assembly of nitric oxide analyzer based on the principle of ozone-mediated chemiluminescence.

NO from gaseous samples reacts with ozone under formation of electrical excited nitrogen dioxide. Emitted light (chemiluminescence) is detected by a photomultiplier tube (PMT). For details see text.

## 6.2 Gas exchange 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 cell 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. In case where two chambers were used in parallel, identical gas flow was conducted through both chambers, only one of them was pulled through the analyzer at a given time, whereas the other one was not measured. The two gas streams were directed to the analyzer or to the waste by means of two electric valves with a self-made electronic timer. 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 CO<sub>2</sub>, 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. The whole device was characterized in the Figure 69.



**Figure 69:** View of the chemiluminescence detection system.

Not shown: vacuum pump with ozone destroyer, lamp and electric 3-way gas switches.

## 7 Northern Blotting

### 7.1 Total RNA isolation

At different time points following infiltration of leaves with cryptogin 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  $\mu$ l  $\text{CHCl}_3$  was added. After a vigorous shaking, the lysate was centrifuged at 12000g for 15 min. Nucleic acids were precipitated with isopropanol (500  $\mu$ 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  $\mu$ 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.

## **7.2 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 iScript™ 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).

## **7.3 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
<i>Taq</i> DNA-Polymerase (10 U/µl)	0.4 µL
H <sub>2</sub> O	39.6 µL

#### Oligo Nucleotide Primers:

Name	Nucleotide Sequence	T <sub>m</sub>
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 µL ethidium bromide after cooling to 50°C. The PCR product (30 µL) 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)

## **7.4 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  $\mu$ 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  $\mu$ l Formamide

100  $\mu$ l 10x MOPS

350  $\mu$ l 37 % Formaldehyde

180  $\mu$ l H<sub>2</sub>O

100  $\mu$ l 80 % Glycerol

80  $\mu$ l 2 % Bromophenol blue solution

10  $\mu$ l 1 % Ethidium bromide

### Formaldehyde-Agarose-Gel

1.5 g Agarose  
90.0 ml H<sub>2</sub>O 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 H<sub>2</sub>O (5 min) and 60 min with 10x SSC.

### 10x SSC

1.5 M NaCl  
0.15 M sodium-Citrate  
pH 7.0 (HCl)

## **7.5 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.

## **7.6 Radioactive labelling 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 µl DNA (25 ng) + 20 µl H<sub>2</sub>O) was denatured (5 min, 100°C) and then mixed with dNTPs (dGTP, dATP, dTTP), 1 µl Klenow-Polymerase (7-12 U, FPLC*pure*<sup>™</sup>; GIBCO, Karlsruhe, Germany), 15 µl 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 µCi

[ $\alpha$ -<sup>32</sup>P]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.

## 7.7 Hybridization of Nucleic Acids

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<sub>2</sub>HPO<sub>4</sub>, 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  $\mu$ ci/ $\mu$ 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.

## 8 Chemicals, enzymes and gases

If not mentioned, all chemicals were received from the companies Biomol (Hamburg, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma (Deisenhofen, Germany). These chemicals were of the highest purity available.

The others chemicals, enzymes and gas, more specifics for our purpose, were purchased from following various suppliers:



**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
tma-PTIO	Alexis, Grünberg, Germany

**Enzymes:**

Catalase	Sigma, Deisenhofen, Germany
Nitrate reductase from corn	Sigma, Deisenhofen, Germany
	NECi, Lake Linden, MI
Nitric oxide synthase, constitutive, rat neuronal, recombinant	Calbiochem, Schwalbach, Germany
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

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## F APPENDIX

### 1 Abbreviations

%	percent
°C	degree Celsius
A	Adenine
ABA	Abscisic Acid
act	actual
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribose
AMP	Adenosine-5'-monophosphate
AO	Alternative Oxidase
ATP	Adenosine-5'-triphosphate
BH <sub>4</sub>	Tetrahydrobiopterin
BSA	Bovine Serum Albumin
C	Cytosine
cADPR	cyclic ADP Ribose
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
cDNA	Complementary DNA
cGMP	cyclic Guanosine 3',5'-Monophosphate
CHX	Cycloheximide
cm	centimeter
CM	Carboxymethylamine
cNR	constitutive Nitrate Reductase
c-PTI	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl
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
DNA	Deoxyribonucleic Acid
dNTP	2'-desoxyribonucleosid-5'-triphosphate
DPI	Diphenyleneiodonium
DTT	1,4-dithiotreitol
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediamine-tetraacetate

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<i>e.g.</i>	<i>exempli gratia</i> (= 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 $\omega$ -nitro-L-arginine methyl ester hydrochloride
L-NIL	L-N <sup>6</sup> -(1-Iminoethyl)-lysine, acetate
L-NMMA	N <sup>G</sup> -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

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MOPS	4-morpholinic-propansulfonic acid
mRNA	messenger RNA
n	Number of samples
N <sub>2</sub>	Nitrogen
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxydized)
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
nL	nanoliter = 10 <sup>-9</sup> L
nM	nanomolar = 10 <sup>-9</sup> M
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 <sup>-12</sup> mol
PMS	Phenasine 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 pirrolidone
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	rounds per minute
RSNO	S-nitrosothiols

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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
<i>Taq</i>	<i>Thermus aquatic</i>
TBE	Tris-borate EDTA
T <sub>m</sub>	melting Temperature
tma-PTIO	3,3,4,4-Tetramethyl-2-trimethylammonio-phenyl-2-imidazoline-3-oxide-1-yl-oxo chloride
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
ZMP	5-Aminoimidazole-4-carboxamide 1-β-D-ribose 5'-monophosphate



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## PUBLICATION LIST

### Publications

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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.

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Würzburg, März 2004

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