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Research article

# The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*

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ABSTRACT

In contrast to the in-depth knowledge concerning nitric oxide (NO) function, our understanding of NO synthesis in plants is still very limited. In view of the above, this paper provides a step by step presentation of the reductive pathway for endogenous NO generation involving nitrate reductase (NR) activity and nitrite implication in potato defense to *Phytophthora infestans*. A biphasic character of NO emission, peaking mainly at 3 and then at 24 hpi, was detected during the hypersensitive response (HR). In avr *P. infestans* potato leaves enhanced *NR* gene and protein expression was tuned with the depletion of nitrate contents and the increase in nitrite supply at 3 hpi. In the same time period a temporary down-regulation of nitrite reductase (*NiR*) and activity was found. The study for the link between NO signaling and HR revealed an up-regulation of used markers of effective defense, i.e. Nonexpressor of *PR* genes (*NPR1*), thioredoxins (*Thx*) and *PR1*, at early time-points (1–3 hpi) upon inoculation. In contrast to the resistant response, in the susceptible one a late overexpression (24–48 hpi) of *NPR1* and *PR1* mRNA levels was observed. Presented data confirmed the importance of nitrite processed by NR in NO generation in inoculated potato leaves. However, based on the pharmacological approach the potential formation of NO from nitrite bypassing the NR activity during HR response to *P. infestans* has also been discussed.



## 1. Introduction

Nitric oxide (NO) is a reactive and simple atomic structure molecule affecting a broad spectrum of physiological and pathophysiological processes in every living organism, including plant cells. Despite the increasing body of knowledge regarding its complex and numerous functions, NO production is always a matter of controversy. Nitric oxide is synthesized in each living cell, but its level is modulated by various endogenous and exogenous stimuli. It may not be excluded that the origin of constitutive NO production in healthy plants differs from the source of the post-stress boosted NO generation.

The biosynthetic pathways of NO in plants may be classified as either oxidative or reductive in operation. The oxidative route involves mammalian-type L-arginine-dependent nitric oxide synthase (NOS)-like activity. The NOS-like activity has been detected in various plant organs, including leaves, roots and nodules, as well as cell cultures (Cueto et al., 1996; Ribeiro et al., 1999; Foissner et al., 2000; Tun et al., 2001). Although homolog genes of mammalian NOS have been identified recently in the genome of photosynthetic organisms including the marine green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi et al., 2010), there is no evidence that higher plants have retained this gene (Hancock, 2012). A recently performed *in silico* search for NOS homologs within 1087 sequenced transcriptomes of land plants revealed no typical NOS sequences, including species, in which the NOS-like activity has been detected (Jeandroz et al., 2016). Polyamines and hydroxylamine are other candidates involved in the oxidative route of NO synthesis in plant cells, activated under normoxic conditions (Tun et al., 2006; Rumer et al., 2009; Wimalasekera et al., 2011).

The reductive pathway towards NO synthesis is dependent on nitrite as a primary substrate and may occur practically within the whole cell environment, including the cytoplasm, mitochondria, chloroplasts, peroxisomes as well as the apoplast (Rőszer, 2014). Up to date assimilatory nitrate reductase (NR) has been considered as the hub enzyme for NO synthesis *via* the reductive route. Under physiological conditions the enzyme reduces nitrate to nitrite at the expense of NAD(P)H, but is also able to catalyze 1-electron transfer

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from NAD(P)H to nitrite, resulting in NO formation (Planchet and Kaiser, 2006). Stöhr et al. (2001) suggested the presence of a plasma membrane bound nitrite: NO-reductase (NI-NOR), which was found to be insensitive to cyanide and unique to NR. The NI-NOR, identified only in tobacco roots and not in leaves, reduced nitrite to NO using reduced cytochrome *c* as an electron donor. NO generation of NI-NOR was comparable to the nitrate reducing activity of a root-specific NR (Stöhr et al., 2001). Nitrite reduction to NO may also occur in the mitochondrial inner membrane. The mitochondrial respiratory chain is able to reduce nitrite to NO at complex III (cytochrome *bc*1) and complex IV (cytochrome-c oxidase); however, this mechanism results in mitochondrial NO generation in cells exposed to hypoxia (Igamberdiev et al., 2010; Gupta and Igamberdiev, 2011; Castello et al., 2006). What is noteworthy, nitrite reductase activity, optimally operative under $O_2$ limitation, has been assigned to the molybdopterin enzyme family including xanthine oxidoreductase and aldehyde oxidase (Weidert et al., 2014), but there is little evidence of its major involvement in plant systems.

An alternative route of NO production involves non-enzymatic NO formation. This type of chemical NO release might occur at the acidic pH of the apoplast, in the presence of ascorbic acid and other reductants (Yamasaki et al., 1999; Bethke et al., 2004; Wang and Hargrove, 2013). Moreover, there is *in vitro* evidence that simultaneous exposure of carotenoids to nitrite and light resulted in NO generation (Cooney et al., 1994).

Several lines of evidence suggested that NR activity is the core source for NO signal production by plants in response to both abiotic and biotic stress factors. The induction of an NR-dependent route of NO synthesis was documented during osmotic stress (Kolbert et al., 2010), water stress (Sang et al., 2008), hypoxia (Benamar et al., 2008; Blokhina and Fagerstedt, 2010) as well as a response to pathogen or elicitor treatment (Yamamoto-Katou et al., 2006; Salgado et al., 2010). Importantly, the *Arabidopsis* mutant *nia1nia2* lacking NR and constantly producing less NO was more susceptible to bacterial or fungal pathogens (Modolo et al., 2005, 2006; Perchepied et al., 2010; Rasul et al., 2012). In addition, Shi and Li (2008) using NR-deficient mutants documented that NO synthesis in response to *Verticillium dahliae* toxins is mostly the origin of the NR pathway and the contribution of the NOS-system appeared to be secondary under pathophysiological conditions.

The mutant or transgenic plants having an altered NO production, while being informative for studying NO-related signaling, are usually different from the wild type plants in terms of many physiological and biochemical features. Thus, direct effects due to an impaired NO synthesis are difficult to distinguish from those caused by metabolic alteration (Leitner et al., 2009). NR deficiency leads to impaired nitrogen assimilation and in consequence affects primary and secondary metabolism. As it was indicated by Modolo et al. (2006), the Arabidopsis *nia1nia2* mutant showed reduced levels of nitrites and amino acids (excluding L-Arginine). These findings revealed a significant role of NR in providing the substrates for NO synthesis both through oxidative and reductive pathways (Salgado et al., 2006). What is more, when NR-deficient (*nia1nia2*) mutants were treated with L-Arginine the total amino acid content increased, whereas *Arabidopsis thaliana* plants were unable to potentiate NO emission and induce hypersensitive response (HR) to *P. syringae* (Oliveira et al., 2009). In turn, when nitrite was supplied into NR-deficient leaves, NO synthesis and HR response to *P. syringae* were recovered (Modolo et al., 2006). In general, the available data revealed a direct effect of NR activity on the delivery of substrates for NO synthesis and an indirect effect on the establishment of plant resistance to the pathogen.

In conclusion, almost 20 years of extensive studies evidencing NO as a fundamental signaling molecule in plant organisms have left a considerable information gap concerning the NO synthesis. Although NOS-like and NR activities are supposed to be the two major enzymatic sources of NO production in plants, the nitrite can be converted into NO by NR-independent route as well (Modolo et al., 2005; Chen et al., 2014). For this reason in the presented paper we focused on the reductive pathway for NO generation involving NR activity and nitrite implication in potato defense to *Phytophthora infestans*. In particular, a combined parallel analysis has been conducted concerning both NR and NiR gene expression with protein activities, nitrate reduction with nitrite leaf tissue concentration during avr *P. infestans* response providing NO overproduction. Finally, looking for the link between pathogen challenged NO generation and HR response we compared the expression of different defense markers, i.e. *NPR1*, *Thx* and *PR1*, in resistant and susceptible potato leaves upon inoculation.

## 2. Materials and methods

### 2.1. Plant material

Sterile potato plants (*Solanum tuberosum* L.) of cv. Bintje − (lacking *R* genes) highly susceptible to isolate 1.3.4.7.10.11. *Phytophthora infestans* and cv. Bzura − (carrying *R* genes derived from *S. demissum*) highly resistant and incompatible to 1.3.4.7.10.11. *P. infestans* were used in the experiments. Potato plants from *in vitro* tissue culture were transferred to soil and they were grown in a growth chamber with 16 h of light (180 μmol $m^{-2} \cdot s^{-1}$) at 18 ± 2 °C and 60% humidity for 4 weeks.

### 2.2. Pathogen culture and inoculation with P. infestans

*Phytophthora infestans* (Mont.) de Bary (1.3.4.7.10.11., isolate MP946), virulent for 'Bintje' and avirulent for 'Bzura', was kindly obtained from the Plant Breeding and Acclimatization Institute, Research Division at Młochów, Poland. Isolate MP946 triggered hypersensitive pointed cell death in the 'Bzura' genotype identified as TUNEL-positive. Potato plants were inoculated by spraying leaves with 5 ml of the oomycete zoospore suspension at a concentration of $2.0 \times 10^5$ per 1 ml of water and then were kept overnight at 100% relative humidity and 18 °C and afterwards they were transferred to a growth chamber.

### 2.3. Measurement of nitric oxide generation

The NO-FL fluorescence from extracts of potato leaves challenged with *P. infestans* was assayed spectrofluorimetrically using a selective nitric oxide sensor (CuFL) similar as described Lim et al. (2006). The copper-complex of FL (2-{2-Chloro-6-hydroxy-5-[2-methylquinolin-8-ylamino)methyl]-3-oxo-3H-xanthen-9-l}benzoic acid) was prepared as 1 mM water stock solution according to the manufacturer's instructions (Strem Chemicals). Leaf tissue (500 mg of fresh weight) was homogenized in 2 ml of 10 mM potassium-phosphate buffer (pH 7.0) and centrifuged at 21,000 × g for 30 min at 4 °C. Then, 100 μl of supernatant was used for NO assay by adding CuFL to the final concentration of 2 μM. After 30 min of incubation in darkness at 25 °C the fluorescence intensity was determined with the Fluorescence Spectrometer Perkin Elmer LS50B (UK) equipped with microtiter plates with 96 wells using 488 nm and 516 nm for excitation and emission, respectively. All data ($F$) are normalized with respect to the emission of CuFL ($F_{CuFL}$). [CuFL] = 2 μM. Each value was expressed as NO-FL fluorescence intensity [$F/F_{CuFL}$].