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Dual role of nitric oxide in *Solanum* spp.–*Oidium neolycopersici* interactions

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ABSTRACT

The role of nitric oxide in the pathogenesis of *Oidium neolycopersici* was studied on leaf discs of three *Solanum* spp. genotypes differing in their susceptibility to powdery mildew infection. The germination of pathogen conidia, development of infection structures and reaction of host tissues were compared for *S. lycopersicum* (susceptible), *S. chmielewskii* (moderately resistant) and *S. habrochaites* f. *glabratum* (highly resistant genotype) in presence of compounds modulating NO levels. The effect of NO donor sodium nitroprusside varied among genotypes and studied time intervals whereas NO scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide accelerated fungal development in all three *Solanum* spp. genotypes. The exposure of leaf discs to NOS inhibitor N^G-nitro-L-arginine methyl ester decreased powdery mildew growth namely in *S. chmielewskii*. Confocal laser scanning microscopy using the fluorescent probe 4-amino-5-(N-methylamino)-2′,7′-difluorofluorescein diacetate localised NO accumulation both in pathogen germ tubes and appressoria and in penetrated cells of resistant genotypes of *S. chmielewskii* and *S. habrochaites* f. *glabratum*. Our results confirm an essential role for NO in powdery mildew pathogenesis including the penetration of biotrophic pathogen and the initiation of hypersensitive reaction, and suggest the contribution of NO to molecular mechanisms of diversity in interactions of *Solanum* spp. with *O. neolycopersici*.

1. Introduction

Nitric oxide (NO), an ubiquitous intra- and intercellular messenger, perform a broad spectrum of regulatory functions in plant growth, ontogeny and responses to multiple stress stimuli (Wendehenne et al., 2001; Lamattina et al., 2003; Neill et al., 2003; del Río et al., 2004). The crucial role of NO in both signalling and defence mechanisms of infected plants has been documented in their interactions with viruses (Durner et al., 1998; Danci et al., 2009), bacteria (Delledonne et al., 1998; Modolo et al., 2005; Mur et al., 2005; Johnson et al., 2008), oomycetes (Sedlářová et al., 2011) and fungi (Tada et al., 2004; Prats et al., 2005; Piterková et al., 2009). NO is indispensable for initiation and progress of plant hypersensitive response (HR), modification of gene expression, and synthesis of pathogenesis-related (PR) proteins (Wendehenne et al., 2004; Zeier et al., 2004; Mur et al., 2006; Zaninotto et al., 2006). The main source of NO in animals is nitric oxide synthase (NOS) while several enzymes were described to produce NO in plants, i.e. NOS-like enzyme, nitrate reductase, and nitrite:NO reductase, in addition to non-enzymatic generation of NO from nitrite (Yamasaki et al., 1999; del Río et al., 2004; Zemojtel et al., 2006; Arasimowicz and Floryszak-Wieczorek, 2007). Despite a decade of intensive research, the origin and function of NO in plants under physiological and stress conditions still awaits to be elucidated (Planchet et al., 2006; Neill et al., 2008; Wilson et al., 2008).

Previous reports revealed an intimate interplay between NO and reactive oxygen species (ROS) during plant–pathogen interactions. Intensive ROS production in the infected plants triggers HR and plant cell wall reinforcement and is involved in microbe destruction (Bolwell and Wojtaszek, 1997; Neill et al., 2002; Wendehenne et al., 2004; Mur et al., 2008; Yoshioka et al., 2009). HR, attributed mainly to race-specific interactions, is conditioned by a rapid accumulation of both ROS (Keller et al., 1998) and NO (Delledonne et al., 1998). Synergistic action of NO and H_2O_2 is believed to orchestrate the localized cell death thus restricting pathogen invasion (Zaninotto et al., 2006), although the mechanism of interaction between the molecules is still a matter of debate (De Gara et al., 2003; Delledonne et al., 2003; Tada et al., 2004; Mur et al., 2006; Wilson et al., 2008). Moreover, both ROS and NO are produced also

Abbreviations: cAMP, cyclic adenosine monophosphate; DAF-FM DA, 4-amino-5-(N-methylamino)-2′,7′-difluorofluorescein diacetate; hpi, hours post inoculation; HR, hypersensitive response; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PR proteins, pathogenesis-related proteins; PTIO, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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by microorganisms, especially those with mycelial growth (Johnson et al., 2008; Prats et al., 2008; Sedlářová et al., 2011). This facilitates active penetration of oomycetes and fungi into host cells. However, the specific role of NO and ROS can vary among pathosystems, influenced by metabolism of the host plant, life strategy of the pathogen, and environmental conditions (Shetty et al., 2008).

Oidium neolycopersici (Kiss et al., 2001), an epiphytic biotrophic pathogen positioned among ascomycete fungi, has caused epidemic infections on glasshouse tomato crops since the 1980s (Jones et al., 2001; Mieslerová and Lebeda, 1999; Mieslerová et al., 2002). Most of tomato cultivars (*Solanum lycopersicum*) are considered highly susceptible to the tomato powdery mildew. However, extensive screening revealed many valuable sources of potential resistance among wild *Solanum* spp. (Lebeda and Mieslerová, 2002). Histological studies revealed that resistant *Solanum* species utilize HR to prevent *O. neolycopersici* spread to non-infected parts of the plant. Though HR does not abolish completely the growth of mycelium, it usually suppresses the pathogen reproduction (Huang et al., 1998; Mieslerová et al., 2004).

Model interactions of *S. lycopersicum* (Amateur), *S. chmielewskii* (LA 2663) and *S. habrochaites* (LA 2128) with *O. neolycopersici* have been studied in our laboratory for several years from anatomical, physiological and molecular perspectives. Previous studies e.g. showed that moderately resistant *S. chmielewskii* expressed HR more intensively than highly resistant *S. habrochaites* (Mieslerová et al., 2004). Timing as well as intensity of antioxidant enzymes activity and ROS production in *Solanum* spp. genotypes during powdery mildew pathogenesis correlated with degree of their resistance (Mlíčková et al., 2004; Tománková et al., 2006). Additionally, production of secondary metabolites (alkaloids, saponins, phenol compounds, etc.) was predicted to influence interactions within this pathosystem (Mieslerová et al., 2004). Recently, we demonstrated NO production by a NOS-like arginine-dependent enzyme related to the activation of both local and systemic resistance mechanisms (Piterková et al., 2009). We hypothesized interaction of NO and H_2O_2 in response to powdery mildew can form molecular basis of *Solanum* spp. resistance to powdery mildew. Preliminary results indicated also NO production by mycelium of *O. neolycopersici* (Piterková et al., 2009), similarly to that reported for barley powdery mildew, *Blumeria graminis* f. sp. *hordei* (Prats et al., 2008) or lettuce downy mildew, *Bremia lactucae* (Sedlářová et al., 2011).

Herein we present the study of NO role over *O. neolycopersici* development on three *Solanum* spp. genotypes with various reaction patterns to powdery mildew, using compounds modulating endogenous NO level in a leaf disc experiments during 72 h post inoculation. Our objective was to determine the relation between increased or decreased NO levels and the development of pathogen structures on leaf discs. To this purpose we tested the hypothesis that: (1) NO is produced both by pathogen and plant cells during various stages of pathogenesis and (2) the effects of NO level modulation are variable among plant genotypes depending on their resistance mechanism to the biotrophic pathogen.

2. Materials and methods

2.1. Plant material

Three genotypes of *Solanum* spp. expressing differential level of resistance to *O. neolycopersici* were used: highly susceptible *Solanum lycopersicum* L. cv. Amateur, moderately resistant *S. chmielewskii* (Rick, Kesickii, Forbes and Holle) Spooner, Anderson and Jansen (LA 2663) and highly resistant *S. habrochaites* S. Knapp & D.M. Spooner f. *glabratum* (LA 2128) (Mieslerová et al., 2004). Seeds were sown on moistened Perlite (Agroperlite, Nový Jičín, Czech Republic). Seedlings were transferred into a garden soil-peat mixture (2:1, v/v) in plastic pots (7 cm in diameter) and grown in a growth chamber with 12-h light/dark cycles with light intensity of 100 μmol photons m^{-2} s^{-1} at 20/18 °C. Plants aged approximately 10 weeks were used for the following experiments.

2.2. Application of compounds modulating NO metabolism

Leaf discs (12 mm in diameter) were cut out of 4th true leaves of tomato plants by a cork borer and laid adaxial side up in Petri dishes (16 discs per dish) with filter paper moistened by 10 ml of one of the following solutions: distilled water (control); 0.1 mM SNP (sodium nitroprusside); 1 mM L-NAME (N^G-nitro-L-arginine methyl ester); 0.1 mM PTIO (2–phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide). All solutions were prepared freshly prior to their application.

2.3. Pathogen isolate, inoculation and incubation

O. neolycopersici Kiss (isolate C-2) from the collection of the Department of Botany, Palacký University in Olomouc, included in the Czech National Collection of Microorganisms (collection number UPOC-FUN-127) was used for the experiments (Mieslerová et al., 2004). The pathogen was maintained and multiplied on plants of susceptible *S. lycopersicum* cv. Amateur aged 5–8 weeks, grown under plastic covers in a growth chamber at 20/18 °C, 12/12 h day/night photoperiod and light intensity of 100 μmol photons m^{-2} s^{-1} (Tománková et al., 2006).

Adaxial side of each leaf disc was inoculated by a surface contact (dusting/tapping) with leaves bearing fresh sporulating mycelium of tomato powdery mildew. The average number of powdery mildew conidia delivered to leaf discs reached 65 ± 15 mm^{-2}. Following inoculation, Petri dishes with leaf discs were incubated in a growth chamber at 20/18 °C and 12 h photoperiod (light/dark).

2.4. Pathogen germination and growth

Leaf discs were collected 8, 24, 48 and 72 h post inoculation (hpi), immersed in 100% acetic acid for 48 h, mounted in glycerol and prior to light microscopy (Olympus BX50 equipped with CCD digital camera Olympus DP70) stained with 1% cotton blue (Lebeda and Reinink, 1994). For each treatment following parameters were studied on four leaf discs per time interval: number of germ tubes per conidia and the length of individual germ tubes. A minimum of 120 conidia, randomly selected on leaf discs, were evaluated per each treatment and time interval. Values are given as mean and standard error.

2.5. Histochemical localization of NO by confocal microscopy

Leaf discs incubated with distilled water or solutions of NO modulators were collected 24, 48, and 72 hpi and incubated in 10 μM solution of DAF-FM DA (4-amino-5-(N-methylamino)-2′,7′-difluorofluorescein diacetate) for 30 min, mounted on microscopic slides and subjected to confocal laser scanning microscopy on Olympus Fluorview 1000 attached to inverted microscope IX81 (Olympus, Japan). Excitation was provided by the 488 nm line of an argon ion laser; a 505 nm dichroic filter and 519 nm longpass emission filter were used. To adjust the right intensity of lasers the samples from control uninoculated plants were examined in the beginning of experiment.

2.6. Statistical analysis

Statistical significance of differences among variants were evaluated by one-way analysis of variance (ANOVA; $P < 0.05$), followed