Local and systemic production of nitric oxide in tomato responses to powdery mildew infection

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SUMMARY

Various genetic and physiological aspects of resistance of *Lycopersicon* spp. to *Oidium neolycopersici* have been reported, but limited information is available on the molecular background of the plant–pathogen interaction. This article reports the changes in nitric oxide (NO) production in three *Lycopersicon* spp. genotypes which show different levels of resistance to tomato powdery mildew. NO production was determined in plant leaf extracts of *L. esculentum* cv. Amateur (susceptible), *L. chmielewskii* (moderately resistant) and *L. hirsutum* f. *glabratum* (highly resistant) by the oxyhaemoglobin method during 216 h post-inoculation. A specific, two-phase increase in NO production was observed in the extracts of infected leaves of moderately and highly resistant genotypes. Moreover, transmission of a systemic response throughout the plant was observed as an increase in NO production within tissues of uninoculated leaves. The results suggest that arginine-dependent enzyme activity was probably the main source of NO in tomato tissues, which was inhibited by competitive reversible and irreversible inhibitors of animal NO synthase, but not by a plant nitrate reductase inhibitor. In resistant tomato genotypes, increased NO production was localized in infected tissues by confocal laser scanning microscopy using the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate. NO production observed in the extracts from pathogen conidia, together with elevated NO production localized in developing pathogen hyphae, demonstrates a complex role of NO in plant–pathogen interactions. Our results are discussed with regard to a possible role of increased NO production in pathogens during pathogenesis, as well as local and systemic plant defence mechanisms.

INTRODUCTION

The induction of the plant defence response in response to stress stimuli is facilitated by a number of signalling pathways. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been shown to be crucial components of the plant stress response (De Gara *et al.*, 2003; Delledonne *et al.*, 2001, 2002; Mur *et al.*, 2008; Wendehenne *et al.*, 2004; Yoshioka *et al.*, 2008; Zaninotto *et al.*, 2006). Although nitric oxide (NO) has been the focus of plant stress physiology and biochemistry research during the past decade, the exact origin, fate and function of NO in plants under different physiological and stress conditions still remain to be determined (Arasimowicz and Floryszak-Wieczorek, 2007; Neill *et al.*, 2008; Planchet and Kaiser, 2006b; Planchet *et al.*, 2006; Wilson *et al.*, 2008). Several enzymes have been shown to produce NO in plants: NO synthase (NOS)-like enzyme, nitrate reductase and nitrite: NO reductase (Arasimowicz and Floryszak-Wieczorek, 2007; Crawford *et al.*, 2006; Guo, 2006; del Río *et al.*, 2004; Zemojtel *et al.*, 2006).

The prominent function of NO in both signalling and resistance of plants infected by pathogens has been well documented in a number of model pathosystems, for example, interactions of plants with viruses (Durner *et al.*, 1998), bacteria (Delledonne *et al.*, 1998; Modolo *et al.*, 2005; Mur *et al.*, 2005), oomycetes (Petřivalský *et al.*, 2007) and fungi (Prats *et al.*, 2005; Tada *et al.*, 2004). Extensive research has focused on the signalling role of NO in the initiation and progress of the plant hypersensitive response (HR), accumulation of pathogenesis-related (PR) proteins and modification of gene expression (Mur *et al.*, 2006; Wendehenne *et al.*, 2004; Zaninotto *et al.*, 2006). HR, attributed especially to race-specific interactions, is conditioned by the rapid accumulation of ROS (Keller *et al.*, 1998) and NO (Delledonne *et al.*, 1998). The synergistic action of NO and H₂O₂ is believed to orchestrate the localized cell death response and restrict pathogen invasion (Zaninotto *et al.*, 2006), although the mechanism of interaction between NO and ROS is still a matter of debate (De Gara *et al.*, 2003; Delledonne *et al.*, 2003; Mur *et al.*, 2006; Tada *et al.*, 2004; Wilson *et al.*, 2008). Direct

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chemical reaction between NO and H_2O_2 produces either highly reactive singlet oxygen or hydroxyl radicals (Noronhadutra *et al.*, 1993), which can induce the processes of cell death. Reaction of NO with O_2^- leads to the formation of a strong oxidant compound, peroxynitrite (ONOO^-), although it seems to be relatively non-toxic to plant cells, in contrast with studies conducted with animal cells. Peroxynitrite is not an essential signal for cell death in soybean cell culture (Delledonne *et al.*, 2001), although it has been reported to induce PR-1 accumulation in tobacco leaves (Durner and Klessig, 1999) and to modulate the cell redox state through protein nitration (Delledonne *et al.*, 2001; Saito *et al.*, 2006). NO is also known to induce a set of plant defence genes, including phenylalanine ammonia-lyase and chalcone synthase, two key enzymes of the phenylpropanoid pathway, leading to the production of secondary compounds, such as phytoalexins and lignin precursors (Dixon, 2001).

Systemic acquired resistance (SAR) in healthy plant tissue is usually activated by cell death in incompatible plant–pathogen interactions of locally infected plants. This triggers the release of short- and long-distance mobile signal molecules, including salicylic acid, methyl salicylate, jasmonic acid and yet poorly defined lipid and peptide effectors (Heil and Ton, 2008; Vlot *et al.*, 2008). Signalling relationships between NO, H_2O_2 and salicylic acid, starting in the vicinity of pathogen attack to the final induction of SAR in distal tissues, have been observed during the development of SAR (Delledonne *et al.*, 2001; Song and Goodman, 2001). The external application of NO induces endogenous accumulation of SA and its conjugates in tobacco, conditioning the establishment of SAR (Durner *et al.*, 1998).

Powdery mildew *Oidium neolyopersici*, a biotrophic epiphytic pathogen, causes serious epidemics, mainly in glasshouse tomato crops in Europe and North America (Kiss *et al.*, 2001; Lebeda and Mieslerová, 1999). According to previous studies, the resistance in the pathosystem *Lycopersicon* spp.–*O. neolyopersici* is conferred by monogenic genes (Bai *et al.*, 2005; Huang *et al.*, 2000; Li *et al.*, 2007). The dominant resistance genes (*Ol-1*, *Ol-3*, *Ol-4*, *Ol-5* and *Ol-6*) confer race-specific resistance by hampering the fungal growth via HR of the host epidermal cells, whereas the recessive gene *ol-2* confers resistance via papilla formation (Bai *et al.*, 2005). Our previous experiments have shown a relationship between ROS production and *Lycopersicon* spp. resistance to tomato powdery mildew (Mlíčková *et al.*, 2004; Tománková *et al.*, 2006). This work aimed to determine the possible role of NO in plant–pathogen interactions as well as the local and systemic resistance of tomato. Three *Lycopersicon* spp. genotypes, *L. esculentum* cv. Amateur (susceptible), *L. chmielewskii* (LA 2663, moderately resistant) and *L. hirsutum* f. *glabratum* (LA 2128, highly resistant) (Mieslerová *et al.*, 2004), were studied during the course of 216 h post-inoculation (hpi) by *O. neolyopersici*.

Fig. 1 Schematic diagram of *Lycopersicon esculentum* cv. Amateur plant with labelled leaves of interest: 1A, 2A, 3A, first, second and third leaves above the inoculation site; 1B, 2B, 3B, first, second and third leaves below the inoculation site.

RESULTS

The study of tomato reactions to powdery mildew infection was focused on the determination of NO production in the leaf extracts, estimated as arginine-dependent NO generation by the oxyhaemoglobin method. Tomato plants at the stage of eight true leaves were used for the experiments; only the fourth true leaf counted from the base up was inoculated with conidia of *O. neolyopersici*. During 216 hpi, NO production was monitored separately in extracts of inoculated leaves and of three leaves above and below the inoculated leaf (Fig. 1).

Local response to infection: NO production in the extracts of inoculated leaves

Significant differences in the extent and timing of the increase in NO production were found between susceptible and resistant tomato genotypes. In the susceptible genotype *L. esculentum* cv. Amateur, elevated NO production was observed only during the early interval following inoculation, at 4–8 hpi (Fig. 2A). However, a two-phase increase in NO production was detected in the leaf extracts of both resistant genotypes (Fig. 2B, C). For *L. chmielewskii* (LA 2663), the first phase was detected between 4 and 72 hpi, with a maximum at 24–48 hpi, whereas, for *L. hirsutum* f. *glabratum* (LA 2128), the first phase culminated at 8 hpi. A pronounced and continual increase in NO production started from 96 hpi for both *L. chmielewskii* and *L. hirsutum*, and lasted up to the end of the studied interval at 216 hpi.

Systemic response to infection: NO production in the extracts of uninoculated leaves

The temporal changes in NO production varied in individual *Lycopersicon* spp. during the course of *O. neolycopersici* patho-

Fig. 2 Time course of relative changes in nitric oxide (NO) production within the extracts of leaves of *Lycopersicon* spp. genotypes inoculated by *Oidium neolycopersici* and in adjacent leaves below and above the inoculated leaf. Data represent the differences between infected and control plants. A, *Lycopersicon esculentum* cv. Amateur (susceptible); B, *L. chmielewskii* (LA 2663) (moderately resistant); C, *L. hirsutum* f. *glabratum* (LA 2128) (resistant). □, adjacent leaf above inoculation site; ●, inoculated leaf; △, adjacent leaf below inoculation site. Results are given as the mean $\pm$ standard deviation ($n = 9$), and values statistically significantly different from the zero time point are marked as * $P = 0.05$ or ** $P = 0.01$.

genesis. For the first time, to our knowledge, we have demonstrated a plant systemic response to pathogen infection in the form of increased NO production in the extracts of uninoculated distal leaves (Figs. 2A–C, 3A–C). In susceptible *L. esculentum*, a peak of NO production was detected in the uninoculated leaf above the inoculated leaf at 8 hpi (Fig. 2A), whereas hardly any difference was recorded in the leaves below the inoculated leaf. No changes were found at later stages of infection (Figs. 2A, 3). However, a biphasic increase in NO production was detected in the moderately resistant and resistant plants, although with different timing. In *L. chmielewskii*, the timing of NO production changes differed between adjacent leaves above and below the inoculated leaf (Fig. 2B). An earlier increase in NO production with a maximum at 4 hpi was observed in the upper adjacent leaf in *L. chmielewskii*, whereas the enhancement of NO production in the lower adjacent leaf continued until the first maximum at 48 hpi, analogous to the inoculated leaf. The timing of the second maximum of NO production was similar for adjacent leaves both above and below the inoculated leaf at 168 hpi. In *L. hirsutum*, the changes recorded in uninoculated leaves close to the infection site were parallel for both the upper and lower leaves, but differed in intensity. The first prominent increase in NO production occurred at 8 hpi in all inoculated and adjacent uninoculated leaves. However, the second maximum occurred at 72 hpi for uninoculated leaves (Fig. 2C), with a subsequent decline at later stages, whereas, in the inoculated leaf, the second increase was found at 168 and 216 hpi. In both resistant tomato genotypes, the elevation of NO production was stronger in the extracts of older (lower) leaves than younger leaves (from the upper part of the plants).

Variation among *Lycopersicon* spp. genotypes with regard to changes in NO production in the extracts of both inoculated and adjacent uninoculated leaves should be interpreted with respect to the resistance mechanisms and dynamics of pathogen growth. Two main phases of powdery mildew development can be recognized: (i) early germ tube development [including conidia germination (3–9 hpi), development of appressoria (6–12 hpi), formation of haustoria (12–24 hpi) and the initiation of intensive mycelium branching (48–72 hpi)]; and (ii) late stages—mycelium expansion or retardation by HR at 72–216 hpi (Fig. 3A–C). In compatible interactions (*L. esculentum*–*O. neolycopersici*), char-

Fig. 3 Increase in nitric oxide (NO) production in the extracts of leaves of *Lycopersicon* spp. genotypes inoculated by *Oidium neolyopersici*, and in three leaves below and above the inoculated leaf. The results are expressed as the difference between infected and control healthy plants at 4 hpi (A), 72 hpi (B) and 216 hpi (C). *Lycopersicon esculentum* cv. Amateur (grey column), *L. chmielewskii* (LA 2663) (dotted column), *L. hirsutum* f. *glabratum* (LA 2128) (black column). 1A, 2A, 3A, first, second and third leaves above the inoculation site; I, inoculated leaf; 1B, 2B, 3B, first, second and third leaves below the inoculation site (see Fig. 1). Results are given as the mean $\pm$ standard deviation ($n = 9$), and values statistically significantly different from the zero time point are marked as * $P = 0.05$ or ** $P = 0.01$.

acterized by rapid pathogen growth and no HR, the increase in NO production in leaf extracts was recorded only during pathogen germination in the inoculated leaf and the upper uninoculated leaves (Fig. 3A). In incompatible interactions (*L. chmielewskii*, *L. hirsutum*–*O. neolyopersici*), typified by the expansion of HR, NO production in leaf extracts increased in the whole plant body with pathogen germination (Fig. 3A). During later stages of

Fig. 4 Relative changes in *in vitro* nitric oxide (NO) production in a conidial extract of the pathogen (A) and in an extract of *Lycopersicon chmielewskii* (LA 2663) inoculated leaves at 24 hpi (B), and the effect of pretreatments with NO-modulating compounds. The extracts were pre-incubated with 1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) (specific NO scavenger), 1 mM *N*^G-nitro-L-arginine methyl ester (L-NAME) or 5 mM aminoguanidine [nitric oxide synthase (NOS) inhibitors] for 5 min prior to the measurement of enzyme activity. In the last variant, L-arginine (NOS substrate) was omitted from the reaction mixture. The data represent the percentage of NO production in pathogen (A) or plant (B) extracts in optimal reaction conditions (100%). The results are given as the mean $\pm$ standard deviation ($n = 3$ independent treatments).

pathogenesis, the reaction varied with the degree of host resistance (Fig. 3B,C). At the end of the studied interval (216 hpi), the enhancement of NO production was detected in the inoculated leaves of both resistant genotypes, and in the moderately resistant *L. chmielewskii* also in uninoculated leaves close to the infected leaf (Fig. 3C).

NO production in the extracts of pathogen infection structures

The oxyhaemoglobin method was used to determine NO production in extracts from *O. neolyopersici* conidia (Fig. 4A). NO production in the extracts of pathogen conidia, used as inoculum, reached 1.0 nmol/s/g fresh weight (FW) (Table 1). Furthermore, an estimation of the contribution of pathogen NO production to the total value measured in the extracts from inoculated leaves was performed. The amount of pathogen

Table 1 Comparison of *in vitro* nitric oxide (NO) production in the leaf extracts of control and inoculated plants (8 hours post-inoculation, hpi) and the extracts of pathogen conidia.

	NO production (nmol/s/g FW)	
	<i>L. esculentum</i> cv. Amateur	<i>L. chmielewskii</i> (LA 2663)
Control leaf	0.146 ± 0.011	0.169 ± 0.013
Inoculated leaf 8 hpi	0.172 ± 0.031	0.256 ± 0.042
Pathogen	0.0010 ± 0.0003	

conidia transferred to leaves of *L. esculentum* cv. Amateur by surface contact during inoculation was estimated to be 1–2 mg by the weight difference, which corresponds to 0.001–0.002 nmol/s of pathogen NO production per gram of FW of the inoculated leaves. At the time of inoculation, this value represents approximately 0.7% of the NO production in the extract of an uninoculated leaf of *L. esculentum* cv. Amateur and 0.6% of the activity in *L. chmielewskii*.

Identification of NO source in pathogen infection structures and inoculated tomato leaves

The identity of NO as the compound reacting with oxyhaemoglobin in the assay was confirmed by the complete loss of production potential when the extract was incubated with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Arginine-dependent enzyme activity as an important source of NO was suggested by a 90% loss of activity in experiments in which the NOS substrate L-arginine was omitted from the reaction mixture (Fig. 4A). The competitive inhibitor of animal NOS, *N*^G-nitro-L-arginine methylester (L-NAME), decreased the activity to 42%, and aminoguanidine, an irreversible inhibitor of animal NOS, to 32%.

The effect of several pretreatments was tested to unveil the NO origin in leaf extracts of *L. chmielewskii* (24 hpi) by the oxyhaemoglobin method (Fig. 4B). *In vitro* NO production in the extracts of inoculated leaves was decreased to 47% by the addition of the NO scavenger cPTIO. Similar results were obtained with the application of L-NAME, which reduced the activity to 43%, whereas aminoguanidine decreased NO production to 27% (Fig. 4A, B). NO production was totally abolished in the reaction mixture in the absence of the NOS substrate L-arginine.

Histochemical localization of NO

In control, inoculated and systemic leaves of *L. esculentum* and *L. chmielewskii* (8–216 hpi), the *in vivo* localization of NO was followed by confocal laser scanning microscopy (Fig. 5). The distribution of NO was studied by incubation with the cell-permeable NO probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Usually, there was no fluorescence signal detected within the plant tissues at 8 hpi (Fig. 5A), or very weak in rare cases for *L. chmielewskii* (Fig. 5E).

However, an intense green fluorescence of the NO probe was found in germinating conidia, germ tubes and appressoria of *O. neolyopersici* (Fig. 5A, E). The specificity of the fluorescence signal was confirmed by pre-incubation of leaf samples with the NO scavenger cPTIO (Fig. 5C, G). The same results were obtained after tissue pre-incubation with the NOS competitive inhibitor L-NAME, which almost completely abolished the fluorescence signal. The spatial distribution of the fluorescence signal inside pathogen invasive structures was demonstrated by '2.5D' software modelling, as illustrated by the volume model in Fig. 6.

No dramatic changes in NO localization were revealed during the course of *O. neolyopersici* pathogenesis, as demonstrated at 168 hpi (Fig. 7) and 216 hpi (Fig. 8). NO was localized within pathogen mycelium (especially the tips of hyphae and haustoria) in susceptible *L. esculentum* cv. Amateur (Figs 7A, 8A), whereas, in *L. chmielewskii*, the fluorescence signal for the NO probe also occurred in epidermal cells either in intimate contact with the pathogen or adjacent to the infection site (Figs 7C, 8C). NO was not detected in any of the adjacent uninoculated leaves below or above the inoculation site by DAF-FM DA staining.

DISCUSSION

In this work, an increase in NO production was found in all *Lycopersicon* spp. genotypes inoculated with *O. neolyopersici*, although in a different manner with regard to intensity and timing. A relationship between the developmental stage of the pathogen, the timing of the increase in NO production and H₂O₂ accumulation, as well as the subsequent HR, has been shown by previous and present experimental data. We have shown previously that key periods of *O. neolyopersici* pathogenesis involve: conidia germination (3–9 hpi); development of appressoria (6–12 hpi); formation of haustoria (12–24 hpi); and the initiation of intensive mycelium branching (48–72 hpi) (Mieslerová *et al.*, 2004; Mlíčková *et al.*, 2004). In all tomato genotypes, the first increase in NOS-like activity is observed at 4–8 hpi, concurrent with the oxidative burst (Tománková *et al.*, 2006). This analogous increase in NO production and accumulation of H₂O₂ within plant tissues is linked to the germination of conidia and the formation of appressoria (Mieslerová *et al.*, 2004). This pattern of increase in NO production is similar to that detected by the oxyhaemoglobin assay in soybean and tobacco cell suspensions challenged with an incompatible strain of *Pseudomonas syringae*, where NO accumulation reaches a maximum approximately

Fig. 5 Histochemical detection of nitric oxide (NO) production in the leaves of *Lycopersicon* spp. inoculated with *Oidium neolycopersici* (8 hpi). Confocal fluorescence (A, C, E, G) and light differential interference contrast (B, D, F, H) micrographs of *L. esculentum* cv. Amateur (A–D) and *L. chmielewskii* (LA 2663) (E–H) leaf tissues incubated with the cell-permeable NO-sensitive fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Control specimens (C, D, G, H) were pre-incubated with the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Infection structures: a, appressorium; c, conidium; gt, germ tube. Bar, 20 μ m.

Fig. 6 Spatial distribution of nitric oxide (NO)-specific fluorescence signal within the germinating conidium of *Oidium neolycopersici* (8 hpi). The '2.5D' volume model was reconstructed from a series of 10 cross-sections in LCS Imaris 5.0.3. software.

1 hpi and is followed by a more intensive second burst of NO at 4–8 hpi (Delledonne *et al.*, 1998). Early NO production, known as the NO burst, seems to be closely related to the process of recognition in race-specific interactions (Bennett *et al.*, 2005; Mur *et al.*, 2006). The second and more intense increase in NO production and H_2O_2 levels is coincident with the period of mycelium branching, as the retardation of pathogen growth on resistant plant leaves can be observed earlier than HR symptoms (Mieslerová *et al.*, 2004; Mlíčková *et al.*, 2004). Similarly, a transient NO burst is observed in barley epidermal cells infected by the powdery mildew (*Blumeria graminis* f. sp. *hordei*) just prior to their HR-associated collapse (Prats *et al.*, 2005). The timing of increased NO production and H_2O_2 production within tissues of resistant tomato genotypes is consistent with the hypothesis of essential cooperation of the two molecules in the HR process, as shown in other pathosystems (Delledonne *et al.*, 2002). This is also supported indirectly by data obtained for the susceptible *L. esculentum* cv. Amateur, where increased NO production was recorded only early after inoculation and H_2O_2 accumulation only during the later phase of *O. neolycopersici* pathogenesis. In a similar manner, an early NO burst and a subsequent wave of NO

Fig. 7 Localization of nitric oxide (NO) at later stages of *Oidium neolycopersici* pathogenesis (168 hpi) on *Lycopersicon esculentum* cv. Amateur (A, B) and *L. chmielewskii* (LA 2663) (C, D). Confocal fluorescence (A, C) and bright-field differential interference contrast (B, D) micrographs of tissues stained with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Infection structures: a, appressorium; c, conidium; gt, germ tube. Bar, 50 μ m.

Fig. 8 Localization of nitric oxide (NO) at late stages of *Oidium neolycopersici* pathogenesis (216 hpi) on *Lycopersicon esculentum* cv. Amateur (A, B) and *L. chmielewskii* (LA 2663) (C, D). Confocal fluorescence (A, C) and bright-field differential interference contrast (B, D) micrographs of tissues stained with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Bar, 50 μ m.

generation enhance the resistance of pelargonium to the necrotrophic pathogen *Botrytis cinerea* (Floryszak-Wieczorek *et al.*, 2007).

The results of a study on the influence of the NO scavenger cPTIO and the superoxide dismutase (SOD)-catalysed dismutation of O_2^- to H_2O_2 has indicated that, in oat leaves, NO and O_2^- may not trigger hypersensitive cell death in infected cells; however, they play an important role in the induction of apoptosis in adjacent cells (Tada *et al.*, 2004). In our host–pathogen interaction, the highest production of O_2^- was detected histochemically in the inoculated leaves of susceptible *L. esculentum* cv. Amateur during the first several hours after inoculation (Mlíčková *et al.*, 2004). The role of the possible synchronous production of both O_2^- and NO by increased NOS-like activity is not so clear in this case. A slightly increased content of O_2^- was also detected in the moderately resistant *L. chmielewskii*, whereas the O_2^- signal was low in the leaf tissues of resistant *L. hirsutum* (Mlíčková *et al.*, 2004). The small amount of O_2^- detected in the tissue of resistant plants might be related to its rapid reaction, with possibly increased amounts of generated NO. Recently, the formation of structural barriers by cell wall modifications has been suggested to be controlled by the balance of NO, H_2O_2 and O_2^- production in the initial phases of the interaction of *L. esculentum* with *Colletotrichum coccodes*, the causal agent of anthracnose (Wang and Higgins, 2005a). The generation of H_2O_2 during the development of *O. neolycopersici* was shown to be strongly associated with HR and papilla formation, and thus involved in tomato resistance to powdery mildew (Li *et al.*, 2007). In contrast with our results, increased levels of NO, decreased H_2O_2 levels and, hence, accelerated infection development were reported in *L. esculentum* (cv. Perkoz), susceptible to the necrotrophic pathogen *Botrytis cinerea* (Malolepsza and Rozalska, 2005).

The study of NO production in tomato leaf extracts was complemented by *in vivo* NO imaging of inoculated and systemic tomato leaves, following incubation of the tissues with the cell-permeable NO fluorescence probe DAF-FM DA. The data on NO distribution acquired by confocal microscopy *in vivo* are generally consistent with the results obtained by the spectroscopic oxyhaemoglobin method *in vitro*. In moderately resistant *L. chmielewskii*, a weak fluorescence signal for NO was detected in a few cells in contact with the pathogen structures at 8 hpi, whereas, at a later stage of the *O. neolycopersici* infection process (216 hpi), more intense fluorescence was recorded. The production of NO in plant cells within infected leaves presumably relates to the activation of local resistance mechanisms that involve an increase in NO production. However, no fluorescence signal was recorded in systemic leaves in which an *in vitro* increase in NO production potential was determined. This could be explained by the hypothesis that, in the systemic response, the increase in NO-generating enzyme activity was not transduced into an increased NO level, or

that the increased NO amount was evenly distributed within the whole leaf tissue and low NO concentrations were not sufficient to produce a detectable fluorescence signal. As for susceptible *L. esculentum*, NO was not localized in host tissues, but was documented within the pathogen mycelium and haustoria (Fig. 8C). *In vivo*-recorded NO production by *O. neolycopersici* was confirmed by the oxyhaemoglobin method in extracts from pathogen conidia (Table 1, Fig. 4A). The involvement of the enzyme with arginine-dependent NO-generating activity in the pathogen production of NO was suggested in experiments in the absence of the NOS substrate L-arginine; moreover, NO production in pathogen extracts was decreased by the application of NOS inhibitors (Fig. 4A). Thus, NO production by the endogenous pathogen was expected to contribute to changes in total NO production measured in homogenized tissues of inoculated leaves (Table 1). To distinguish the NO production of the pathogen from that originating in plant leaves, a measurement performed on the separate extracts of both partners is required. Unfortunately, we were unable to separate the growing mycelium without damage, as the biotrophic pathogen lives in intimate contact with host tissues. At least for the early stage after inoculation, the contribution of pathogen conidia was estimated from the specific NO-generating activities and the mean mass of pathogen conidia transferred during inoculation, which seems to represent 0.6–0.7% of total NO production. This is very low and the results of confocal microscopy indicate an intense increase in NO production within the mycelium during the infection process. Unfortunately, this could not be measured *in vitro* as it is not possible to cultivate viable biotrophs on artificial medium for longer time periods. However, a notable conclusion is that the specific enzyme NO-generating activity, when expressed per grams of FW, is one order of magnitude higher in pathogen conidia in comparison with untreated leaves. This may explain why an NO-positive signal was observed in pathogen structures, but was not detectable in tomato leaves using the DAF-FM DA probe (Fig. 5).

Our findings indicate the importance of endogenous NO production during powdery mildew development, as already reported for lettuce downy mildew, *Bremia lactucae* (Petřivalský *et al.*, 2007). Similarly, exogenous application of NO delayed the germination of *Colletotrichum coccodes* conidia, and treatment with NO inhibitors accelerated germination, suggesting that NO may have a regulatory effect on the processes of fungal pathogen germination (Wang and Higgins, 2005b). NO produced by *Blumeria graminis* f. sp. *hordei* was detected recently by 4,5-diaminofluorescein diacetate (DAF-2 DA) and confocal microscopy, and was shown to be a pathogenesis determinant on barley required for the formation of fungal appressoria (Prats *et al.*, 2008). Currently, we are addressing this issue on the model system of tomato leaf discs to investigate specific effects of various NOS inhibitors and NO scavengers or donors on

O. neolycopersici development. However, based on our experience, as well as discussions on the appropriate methodology (Jourdain *et al.*, 2005; Planchet and Kaiser, 2006a; Planchet *et al.*, 2006), we strongly suggest more careful interpretation of the data obtained exclusively by confocal microscopy using diaminofluorescein derivatives.

In vitro NO production in the extracts of uninoculated leaves was related to the level of tomato genotype resistance, and points to a possible role of NO in the process of systemic signal transduction throughout *Lycopersicon* spp. plants. The biphasic timing of the increase in NO production seems to be characteristic only for moderately resistant and highly resistant tomatoes. The second phase of the NO production increase in the extracts of uninoculated leaves shifts to earlier time intervals in highly resistant *L. hirsutum* compared with moderately resistant *L. chmielewskii*. This conditions the presumption of NO participation, especially in the initiation of resistance mechanisms, which are triggered rapidly by *L. hirsutum*. However, the moderately resistant genotype *L. chmielewskii* showed increased NO production in the extracts of adjacent leaves compared with the inoculated leaf at 216 hpi also, when no systemic response was recorded in the other genotypes (Fig. 3C). In both moderately and highly resistant genotypes, the increase in NO production was detectable at 4 hpi in relatively long-distance plant organs, up to the third leaf above and below the inoculated leaf (Fig. 3). These results support the hypothesis that the capacity to rapidly and continuously increase local and systemic NO production by an arginine-dependent NO-generating enzyme, as a response to pathogen challenge, forms a part of the molecular basis of tomato resistance to biotrophic fungi, at least to powdery mildew. NO is likely to play both signalling and defence roles in the systemic resistance of tomato plants. NO has been reported to be involved in phytoalexin accumulation in potato tubers (Nori-take *et al.*, 1996), and the application of NO triggered the biosynthesis of antimicrobial flavonoids in elicitor-treated soybean cotyledons (Modolo *et al.*, 2002). Systemic accumulation of phytoalexins is one of the defence mechanisms suggested for *L. hirsutum* (Mansfield, 2000).

This study did not address directly the nature and fate of long-distance signals transferred from inoculated to distal uninoculated parts of a plant under pathogen attack. In some host-pathogen interactions, it has been shown that interplay between NO, salicylic acid and its conjugates plays a fundamental role in SAR (Durner *et al.*, 1998; Song and Goodman, 2001), although the underlying mechanism has not been elucidated fully. Tomato plants show spiral phyllotaxy, and the third leaf above is the nearest orthostichous leaf to the inoculated leaf. Indeed, at 4 hpi, the highest systemic increase in NOS-like activity in susceptible and moderately resistant genotypes was observed in the third leaf above the inoculated leaf, whereas the resistant geno-

type showed an even stronger response in all three leaves below the inoculation site (Fig. 3A). At later time intervals, the relationship of the systemic response to vascular architecture is much less clear and deserves further investigation. Similarly, the systemic induction of proteinase inhibitor on tomato leaf wounding was strongest in the third leaf above and lowest in the first leaf above the wounded leaf (Oriens *et al.*, 2000). Concerning the downward movement of systemic signals, electrical signals have been described as important mediators of tomato systemic response in both directions, as demonstrated for heat stimuli and mechanical wounding (Heil and Ton, 2008; Peña-Cortés *et al.*, 1995; Stankovic and Davies, 1998).

Several experiments were performed in order to confirm the involvement of the NO-generating enzyme as the source of increased NO production after tomato inoculation. Enzyme and non-enzyme source(s) of NO in plant cells have been the subject of a long-lasting debate with controversial results published repeatedly (Crawford *et al.*, 2006; Guo, 2006; Planchet and Kaiser, 2006b; del Río *et al.*, 2004; Zemojtel *et al.*, 2006). Using the specific NO scavenger cPTIO, we aimed to confirm the reliability of our experimental method based on the interaction of NO with oxyhaemoglobin. Even at high cPTIO concentrations in reaction buffer (up to 1 mM), we did not observe complete inhibition of methaemoglobin production. This could be explained by the much higher rate constant for the NO-oxyhaemoglobin reaction than the NO-cPTIO reaction, and therefore much more efficient scavenging of NO by oxyhaemoglobin in the reaction buffer. As reported previously, NO-mediated oxidation of oxyhaemoglobin to methaemoglobin proceeds with a specific rate constant of $3.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Gow *et al.*, 1999), whereas the rate constant for the reaction of nitronyl nitroxides with cPTIO has been reported in the range $(0.5\text{--}16) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Goldstein *et al.*, 2003). In our host-pathogen interaction, NO is likely to be synthesized, in major part, by an arginine- and NADPH-dependent enzyme(s) that could be inhibited by pretreatment with the competitive inhibitor of animal NOS, L-NAME, and the irreversible inhibitor of animal NOS, aminoguanidine. The involvement of arginine-dependent enzyme activity in NO production was confirmed by the results obtained in the absence of L-arginine and NADPH in the reaction mixture. Similar results to those obtained by oxyhaemoglobin assay were determined by the fluorimetric method using DAF-FM DA, both with respect to differences between the activity values of control and infected plants, and to their sensitivity to the NOS scavenger cPTIO and NOS inhibitors L-NAME and aminoguanidine (data not shown). However, the participation of other enzymatic and non-enzymatic sources in basal or increased NO production in tomato leaves cannot be excluded. We can conclude that the identification of NO sources in *O. neolycopersici* pathogenesis on tomato deserves further detailed study, with special focus on the nature and localization of NO-generating enzyme activities in both leaf

and pathogen cells, and the evaluation of the possible involvement of other NO sources.

To summarize the results discussed above, our study has confirmed the role of increased NO production, probably generated in major part by an arginine-dependent enzyme activity, in the model pathosystem *Lycopersicon* spp.–*O. neolycopersici*. For the first time, we have demonstrated a systemic plant response to biotrophic pathogen infection in the form of increased NO production *in vitro* in the extracts of adjacent and distant uninoculated leaves. The possible synergistic action of NO and H₂O₂ in the tomato response to powdery mildew can be hypothesized on the basis of the results presented here and previous investigations focused on ROS metabolism in the plant–pathogen interaction (Mlíčková *et al.*, 2004). In resistant genotypes, increased NO production was observed on infection, in parallel with increased amounts of H₂O₂, and in two specific time periods in both inoculated and distant uninoculated leaves. Thus, NO and H₂O₂ are likely to form an important part of the molecular mechanism of tomato resistance to *O. neolycopersici*.

EXPERIMENTAL PROCEDURES

Pathogen isolate

Oidium neolycopersici Kiss (isolate C-2) from the Czech National Collection of Microorganisms UPOC (Olomouc) was used for the experiments. The isolate originates from natural infections of *L. esculentum* cv. Lucy plants grown in glasshouses of the State Phytosanitary Administration (SPA, Olomouc, Czech Republic) (Mieslerová *et al.*, 2004). The pathogen was maintained and multiplied on 5–8-week-old plants of susceptible *L. esculentum* cv. Amateur, grown under plastic covers in a growth chamber with a 12-h photoperiod (light intensity, 100 µmol/m²) and day/night temperature of 20/18 °C (Tománková *et al.*, 2006).

Plant material

Three *Lycopersicon* spp. genotypes, *L. esculentum* (syn. *Solanum lycopersicum*) cv. Amateur, *L. chmielewskii* (*S. chmielewskii*) (LA 2663), *L. hirsutum* (*S. habrochaites*) f. *glabratum* (LA 2128), expressing differential phenotypes of resistance to *O. neolycopersici* (Mieslerová *et al.*, 2004), were studied. Seeds were sown on moistened Perlite (Agroperlite, Nový Jičín, Czech Republic) and seedlings were transferred to a garden soil–peat mixture (2 : 1, v/v) in plastic pots (7 cm in diameter). Plants were grown in a growth chamber with a 12-h photoperiod (light intensity, 100 µmol/m²) and day/night temperature of 20/18 °C. Plants aged approximately 10 weeks (at the stage of eight true leaves, Fig. 1) were subjected to the following experiments.

Procedures of inoculation, incubation and sample collection

Freshly sporulating mycelium (8 days post-inoculation) of *O. neolycopersici* (isolate C-2) was used as the source of inoculum. The fourth oldest true leaf per plant was inoculated by surface contact (dusting/tapping) with conidia of *O. neolycopersici*. Both inoculated and control plants of each genotype were kept under plastic covers in a growth chamber at 20/18 °C with a 12-h/12-h light/dark photoperiod. The inoculated leaf as well as three leaves above and three leaves below the inoculated leaf were collected separately at 4, 8, 24, 48, 72, 96, 168 and 216 hpi, immediately frozen in liquid nitrogen and stored at –80 °C. Plant growing and inoculation procedures were repeated in three independent experiments, and for each data point corresponding leaves from three plants were collected.

Preparation of plant extracts

Frozen leaf tissues were homogenized in chilled mortar with ice-cold 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.5) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% polyvinylpyrrolidone and 10% glycerol in a ratio of 1 : 2 (w/v). Prior to homogenization, 5 mM dithiothreitol (DTT), 2 mM aprotinin and 2 mM phenylmethylsulphonyl fluoride were added to the extraction buffer. Leaf homogenates were centrifuged at 16 000 *g* for 15 min at 4 °C, and the supernatants were used immediately for the determination of NOS-like activity.

Preparation of pathogen extracts

Extracts were prepared from frozen *O. neolycopersici* conidia dabbed off 50 leaves of *L. esculentum* cv. Amateur with freshly sporulating mycelium (8 dpi). Approximately 50 mg of pathogen conidia was collected, frozen in liquid nitrogen and stored at –80 °C. Conidia were processed as described for plant tissues, except that homogenization with HEPES was performed in the ratio of 1 : 10 (w/v).

Pretreatment of leaf and pathogen extracts

Prior to the measurement of NOS-like activity by the oxyhaemoglobin method, the extracts were pre-incubated with 1 mM cPTIO, 1 mM L-NAME or 5 mM aminoguanidine for 5 min.

Determination of NO production

NO production in plant or pathogen extracts was determined spectrophotometrically by the oxyhaemoglobin method based on the reaction of NO with oxyhaemoglobin to yield methaemoglobin and nitrate (Hevel and Marletta, 1994). The reaction medium

contained 10 μM oxyhaemoglobin, 1 mM L-arginine, 1 mM CaCl_2 , 1 mM Mg^{2+} , 1 μM calmodulin, 140 μM NADPH, 1 μM flavin adenine dinucleotide (FAD), 1 μM flavin mononucleotide (FMN) and 10 μM tetrahydrobiopterin in 100 mM HEPES buffer, pH 7.5. Norvaline as a competitive inhibitor of potentially interfering tomato arginase was added at a final concentration of 1 mM. SOD (50 U) and catalase (100 U) were added to prevent interference by O_2^- and H_2O_2 . All measurements were performed at 37 °C, and changes in absorbance were followed at $\lambda = 401$ nm every 15 s for 10 min on a Synergy HT microplate reader (Biotek, USA). All measured values were subtracted from the blank values, where the extracts were replaced in the reaction mixture by the same volume of extraction buffer. For each variant, three measurements for each of the above-mentioned three independent sets of samples were conducted, i.e. nine values were obtained. The data are presented as the mean $\pm$ standard deviation.

NO production in the extracts was determined spectrophotometrically also using the NO probe DAF-FM DA. The reaction medium contained 10 μM DAF-FM DA, 1 mM L-arginine, 1 mM CaCl_2 , 1 mM Mg^{2+} , 1 μM calmodulin, 140 μM NADPH, 1 μM FAD, 1 μM FMN, 10 μM tetrahydrobiopterin, 1 mM norvaline, superoxide dismutase (50 U) and catalase (100 U) in 100 mM HEPES buffer, pH 7.5. Fluorescence was measured in 10-min intervals for 1 h in the dark at 37 °C by a Synergy HT microplate reader (Biotek; excitation filter, 485/20 nm; emission filter, 516/20 nm).

Histochemical localization of NO

Pieces of tissue (approximately 1×1 mm) were cut off the control and inoculated leaves of *L. esculentum* cv. Amateur and *L. chmielewskii* (LA 2663) at 8, 24, 48, 72, 168 and 216 hpi. Samples were incubated in a solution of the NO probe 10 μM DAF-FM DA for 30 min, mounted on microscopic slides and subjected to confocal laser scanning microscopy (Fluoview 1000 attached to an inverted microscope IX81; Olympus C&S, Czech Republic). Excitation was provided by a 488-nm line of an argon ion laser. Dye emission was recorded using a 505–525-nm band-pass filter. To adjust the correct intensity of the lasers, samples from control uninoculated plants were examined at the beginning of the experiment. Leaf samples treated for 30 min with 0.1 mM CPTIO prior to staining served as negative control. To exclude sources of NO other than the enzyme with NOS-like activity, the tissues were pretreated with 10 mM L-NAME (competitive inhibitor of animal NOS) and 10 mM aminoguanidine (irreversible inhibitor of animal forms of NOS) for 30 min before staining.

Digital image processing

The spatial distribution of the NO-positive signal within samples was studied by '2.5D' reconstructions from cross-section series in LCS Imaris 5.0.3. software (Bitplane, Switzerland).

Statistical analysis

The experimental data were analysed by the one-way analysis of variance mode of the commercial software Statistica CZ v.8.0. The homogeneity of experimental groups at individual time points was compared by *post hoc* analysis of the corresponding mean values by Fisher's least statistical difference procedure. The significantly different values at the 0.05 and 0.01 probability levels are marked in the figures by single and double asterisks, respectively.

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