Review

Nitric oxide: an effective weapon of the plant or the pathogen?MAGDALENA ARASIMOWICZ-JELONEK^{1,*} AND JOLANTA FLORYSZAK-WIECZOREK²¹Department of Plant Ecophysiology, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland²Department of Plant Physiology, Poznan University of Life Sciences, Wołynska 35, 60-637 Poznan, Poland**SUMMARY**

An explosion of research in plant nitric oxide (NO) biology during the last two decades has revealed that NO is a key signal involved in plant development, abiotic stress responses and plant immunity. During the course of evolutionary changes, microorganisms parasitizing plants have developed highly effective offensive strategies, in which NO also seems to be implicated. NO production has been demonstrated in several plant pathogens, including fungi, but the origin of NO seems to be as puzzling as in plants. So far, published studies have been spread over multiple species of pathogenic microorganisms in various developmental stages; however, the data clearly indicate that pathogen-derived NO is an important regulatory molecule involved not only in developmental processes, but also in pathogen virulence and its survival in the host. This review also focuses on the search for potential mechanisms by which pathogens convert NO messages into a physiological response or detoxify both endo- and exogenous NO. Finally, taking into account the data available from model bacteria and yeast, a basic draft for the mode of NO action in phytopathogenic microorganisms is proposed.

INTRODUCTION

The discovery of Furchgott, Ignarro and Murad in the late 1990s made nitric oxide (NO) one of the most ubiquitous endogenous molecules implicated in the signalling biochemistry of living organisms under both physiological and pathophysiological conditions. In human and animal organisms, this short-lived gas is engaged in the regulation of the cardiovascular, nervous and immune systems (Calabrese *et al.*, 2007). The first study related to plant organisms demonstrated that herbicide-treated soybean leaves form and release NO_(x), including NO (Kleper, 1979). Further research on the role of NO in plants concerned predominantly the toxic action of various nitric oxides (NO₂, N₂O₃, NO₂⁻, NO₃⁻) on the photosynthetic apparatus and chlorophyll levels in forest tree species and plants in parks or industrial areas (Mill and Bennett, 1970). Ground-breaking studies showing NO as a regulating agent

during plant defence appeared as late as 1998 (Delledonne *et al.*, 1998; Durner *et al.*, 1998) and resulted in an explosion of research in the wider area of plant NO biology.

In the battle between plants and pathogens, the most important role is played by the speed of response of the opponents. So far, the role of NO in plant–pathogen interactions has been analysed mainly from the viewpoint of the host. The importance of NO in plant resistance has been well documented. Pharmacological, biochemical and genetic approaches have provided evidence that an early NO burst in plant cells after pathogen attack functions as a messenger in gene-for-gene defence responses and as a general key factor associated with basal resistance in various plant–pathogen systems (e.g. Asai and Yoshioka, 2009; Mur *et al.*, 2006; Schlicht and Kombrink, 2013; Zeier *et al.*, 2004). The above-mentioned topic has been reviewed extensively in many excellent articles (e.g. Bellin *et al.*, 2012; Leitner *et al.*, 2009; Mur *et al.*, 2006); therefore, in this article, we have attempted to discuss the mysterious role of NO from the viewpoint of the pathogen.

During the course of evolutionary changes, microorganisms parasitizing other organisms have implicated NO in highly effective, constitutive and inducible mechanisms of offensive strategies. For example, in endothelial cells, pathogenic microbes and viruses may induce S-nitrosylation of membrane receptors to facilitate cellular entry (Wang *et al.*, 2006). Moreover, there is evidence that NO generated by microorganisms may be responsible for the activation/deactivation of phytotoxins (Kers *et al.*, 2004; Wach *et al.*, 2005). This presents an interesting challenge, as the same molecule could favour both the invader and the host. To clarify the Janus-faced nature of NO effects in plants, Yamasaki (2004, 2005) proposed a concept introducing an evolutionary aspect of this dual relationship. According to this concept, in the beginning, the hostile invader attacked living organisms causing harmful effects by the elicitation of unfavourable exogenous elements, e.g. NO. Later, the living organisms developed defence responses to the invader and evolved tolerance mechanisms against it. Finally, the harmful element (NO) started to play new roles in living organisms when they acquired the ability to actively produce agents for their own needs or even to colonize other living targets. This concept explains the dualism of NO in its effects on living organisms, i.e. harmful and beneficial effects in the same organism (Yamasaki, 2005). The concept also places pathogen invaders at the earliest stage of evolutionary changes, suggesting

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that they first used NO as an efficient weapon to conquer host plants. As stated by Steenbergen *et al.* (2001), it is likely that the ability of pathogenic fungi to combat host–pathogen defences evolved through ancient interactions between fungi and phagocytic amoeba (Tillmann *et al.*, 2011).

Despite extensive research on NO synthesis and signalling processes in human, animal and plant systems, our understanding of NO in microorganisms is very limited. In general, little is known about NO metabolism in plant pathogenic microorganisms, especially in fungi and fungus-like organisms (Floryszak-Wieczorek and Arasimowicz-Jelonek, 2010). However, the signalling mode of NO action seems to involve mechanisms well defined in other living systems, i.e. the cyclic guanosine monophosphate (cGMP) pathway, S-nitrosylation and nitration. Without fail, the collected experimental data provide grounds for the suspicion that NO functions as an effective weapon used by multiple genera of pathogens.

NO PRODUCTION IN BACTERIA

Bacteria are able to produce NO by the oxidation of L-arginine to L-citrulline with the participation of the bacterial nitric oxide synthase (bNOS) enzyme. Structurally, mammalian-type NOSs are dimeric proteins that contain an N-terminal oxygenase domain and a C-terminal flavoprotein reductase domain (Alderton *et al.*, 2001). bNOSs lack the essential reductase domain, which casts doubt on the ability to produce NO by bacteria in nature. However, a relatively slight structural NOS diversity of the NOS oxygenase domain, found throughout prokaryotes and eukaryotes, indicates that it might constitute a core of the enzyme (Correa-Aragunde *et al.*, 2013). Moreover, *in vitro* studies have shown that the *Bacillus subtilis* flavodoxins (YkuN and YkuP) can serve the role of redox partners to the bNOS oxygenase domain to support NO synthesis (Wang *et al.*, 2007). In addition, Gusarov *et al.* (2008) found that bNOSs use redundant cellular reductases as electron donors to produce NO *in vivo*. Interestingly, bacterial proteins homologous to eukaryotic NOSs have been identified and characterized mainly in Gram-positive microbes and archaea (Wang and Ruby, 2011). The *Streptomyces* NOSs additionally contain an N-terminal zinc site in which one of the two conserved cysteine (Cys) residues is replaced by a histidine (His) residue (Kers *et al.*, 2004).

In contrast with most prokaryotes, *Sorangium cellulosum*, a Gram-negative bacterium, has a covalently attached reductase module (Schneiker *et al.*, 2007). The domain architecture includes an N-terminal region of unknown function, followed by a predicted Fe₂S₂ cluster, flavin adenine dinucleotide (FAD)- and nicotinamide adenine dinucleotide (NAD)-binding motifs (Sudhamsu and Crane, 2009). A detailed comparison of animal NOS structure with NOSs from prokaryotes was recently presented by Correa-Aragunde *et al.* (2013). Importantly, the functions of bNOS are still being defined.

Genetic comparisons indicate that bNOS-dependent NO production is possible in many common associated plant genera, including *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Bacillus*, *Geobacillus* and *Paenibacillus* (Cohen *et al.*, 2010). Using cultures of both the wild-type strain of *Streptomyces turgidiscabies* Car8 and the *nos* deletion strain Car8Δ*nos*, Johnson *et al.* (2008) provided direct evidence for bNOS-dependent NO production in plant-pathogenic bacteria. Interestingly, bNOS activity was found to be up-regulated in both *S. turgidiscabies* and *Rhodococcus* strain APG1 in response to plant-derived disaccharides (Cohen and Yamasaki, 2003; Johnson *et al.*, 2008), suggesting that bNOS is activated in the presence of plant tissue. Moreover, L-arginine-dependent NO generation was found in free living cells of the rhizobium *Sinorhizobium meliloti* grown under aerobic conditions (Pii *et al.*, 2007).

Another, main, route for NO generation in free bacteria is the process of denitrification (Zumft, 1997). The classical denitrification pathway is a multiple step reduction of nitrate (NO₃⁻) to nitrogenous gas (N₂) involving the activity of nitrate reductase (NR), nitrite reductase (NiR), NO reductase (NoR) and N₂O reductase (Meilhoc *et al.*, 2011). NO is produced as a free intermediate by NiR.

NO production via the denitrification pathway during plant pathogenic conditions has not been characterized; however, the activation of various truncated denitrification pathways has been identified in several human pathogens (Mocca and Wang, 2012). Furthermore, the formation of NO by the bacterial partner during the legume–rhizobium symbiosis has been reported (Horchani *et al.*, 2011). Pharmacological and mutant approaches have revealed that rhizobial denitrification contributes to NO generation in bacteroids, particularly under hypoxia (Meakin *et al.*, 2007; Sanchez *et al.*, 2010). Moreover, Horchani *et al.* (2011) have shown that approximately one-third of the NO generated by *Medicago truncatula*–*Si. meliloti* nodules is produced by the bacteroid denitrification pathway.

NO PRODUCTION IN FUNGI AND FUNGUS-LIKE ORGANISMS

Although NO production has been proven in plant pathogens, including fungi and fungus-like organisms (Table 1), the origin of NO seems to be unclear, as it is in plants. The organisms cope with a broad range of potential NO sources, which appear to be reductive or oxidative in operation. Fungi do not contain NOS-like sequences in their genomes, except for some fungal species from the genus *Aspergillus* (*A. flavus*, *A. oryzae* and *A. niger*) and *Glomerella graminicola* (Turrión-Gómez and Benito, 2011). However, it has been suggested that NO synthesis in fungi is derived from an L-arginine-dependent pathway using a complex NOS-like system. The first NOS-dependent NO production was demonstrated in the model yeast *Saccharomyces cerevisiae*

Table 1 Nitric oxide (NO) production in plant pathogens.

Phytopathogen	Disease	Documented NO source	Potential function	Reference
Fungi				
<i>Botrytis cinerea</i>	Grey mould	NO ₂ ⁻ Unknown	Development Plant–pathogen interaction	van Baarlen <i>et al.</i> (2004); Conrath <i>et al.</i> (2004); Floryszak-Wieczorek <i>et al.</i> (2007); Turrión-Gómez and Benito (2011)
<i>Colletotrichum coccodes</i>	Anthraxnose	NOS-like	Development	Wang and Higgins (2005)
<i>Blumeria graminis</i>	Powdery mildew	NOS-like	Development Plant–pathogen interaction	Prats <i>et al.</i> (2008)
<i>Cryphonectria parasitica</i>	Chestnut blight	NOS-like	Development	Gong <i>et al.</i> (2007)
<i>Magnaporthe oryzae</i>	Rice blast	Unknown	Development Plant–pathogen interaction	Samalova <i>et al.</i> (2013)
<i>Fusarium</i> sp.	Fusariose	NO ₂ ⁻	Unknown	Conrath <i>et al.</i> (2004)
Oomycete				
<i>Pythium</i> sp.	Water moulds	NO ₂ ⁻	Unknown	Conrath <i>et al.</i> (2004)
Bacteria				
<i>Streptomyces</i> sp.		bNOS	Plant–pathogen interaction	Johnson <i>et al.</i> (2008)

bNOS, bacterial nitric oxide synthase.

(Kanadia *et al.*, 1998). Moreover, the activity of a NOS-like enzyme has been confirmed by measuring citrulline formation from ³H-labelled arginine in the fungi *Neurospora crassa*, *Phycomyces blakesleeanus* and *Coniothyrium minitans* (Li *et al.*, 2010; Maier *et al.*, 2001; Ninnemann and Maier, 1996). Unlike plants, which do not synthesize tetrahydrobiopterin (BH₄), but convert its first intermediate (i.e. dihydroneopterin triphosphate, H₂-NTP) into folate (Stanger, 2002; Wang *et al.*, 2011), both the ascomycete *N. crassa* and zygomycete *P. blakesleeanus* are equipped with BH₄, a typical mammalian NOS cofactor essential for NO synthesis (Maier and Ninnemann, 1995).

Gong *et al.* (2007) demonstrated that conidiation of *C. minitans* was suppressed by a highly efficient inhibitor of NOS activity, i.e. N-nitro-L-arginine methyl ester (L-NAME). Moreover, the insertional disruption of *CMCPS1* encoding L-arginine-specific carbamoyl-phosphate synthase blocked the biosynthesis of L-arginine and subsequently limited NO release in *C. minitans*, indicating the existence of the NOS-like enzyme in this fungus (Gong *et al.*, 2007). A global microarray analysis of the biocontrol fungus *Trichoderma harzianum* revealed the up-regulation of the gene coding for acetylornithine aminotransferase (AcOAT) *in planta* (Samolski *et al.*, 2009). As AcOAT is a pyridoxal phosphate-dependent enzyme involved in arginine biosynthesis, it could imply NO production by the fungus via the L-arginine-dependent pathway. A NOS-like activity was also detected throughout sporulation of the aquatic fungus *Blastocladiella emersonii*, as the enzyme activity dropped significantly in response to NOS inhibitors, i.e. L-NAME and 1-[2-(trifluoromethyl)phenyl]imidazole (TRIM). In addition, NOS assays carried out in the presence of ethylene glycol tetraacetic acid (EGTA) showed a decreased enzyme activity, suggesting the involvement of calcium ions in fungal NOS-like activation (Vieira *et al.*, 2009). L-NAME was also highly

efficient in suppressing NO synthesis in *Blumeria graminis*, a biotrophic fungus causing barley powdery mildew (Prats *et al.*, 2008). In turn, blocking of NO synthesis in the anthracnose pathogen *Colletotrichum coccodes* by various NOS inhibitors suggests that the fungal isoform of NOS may be biochemically similar to mammalian constitutive NOS (Wang and Higgins, 2005). Remarkable differences in NOS-like activity were stressed by Li *et al.* (2010). It ranges from 10 pmol/mg/min in *P. blakesleeanus* mycelia (Ninnemann and Maier, 1996) to 500 pmol/mg/min in *Flammulina velutipes* fruiting bodies (Song *et al.*, 2000). Furthermore, the activity is strictly dependent on the fungus developmental stage, as the highest level of NOS activity, amounting to 20 pmol/min/mg in *C. minitans*, was recorded during conidiation (Li *et al.*, 2010).

However, L-NAME applied to the necrotroph *Botrytis cinerea* growing on medium as well as *in planta* did not diminish the production of NO (Turrión-Gómez and Benito, 2011). Moreover, when searching for the source of NO production through NR, *B. cinerea* was incubated with NO₃⁻; however, no NO signal was detected, suggesting that a physiological and genetic system other than NOS and NR is responsible for NO production in this pathogen (Turrión-Gómez and Benito, 2011).

Although informative, the use of NOS inhibitors in looking for NOS activity in fungi does not provide an unequivocal confirmation for the presence of the enzyme, as these chemicals can also inhibit other biosynthetic pathways (Planchet and Kaiser, 2006). Moreover, the common use of citrulline-based assays casts doubt on the presence of NOS, as interference from argininosuccinate, generated by argininosuccinate lyase, has been reported (Tischner *et al.*, 2007).

More recently, using genetic approaches and bioinformatics tools, Samalova *et al.* (2013) demonstrated that NO synthesis in the hemibiotrophic ascomycete *Magnaporthe oryzae* is not

associated with either a nitrite-dependent or arginine-dependent pathway. By creating mutants with single and double knockout of genes potentially involved in NO synthesis, including NOS-like (*NOL2* and *NOL3*), nitrate (*NIA1*) and nitrite reductase (*NII1*) genes, the authors found that NO is not generated by the candidate proteins. Castello *et al.* (2006) showed that yeast cell mitochondria were capable of NO synthesis independent of NOS-like activity, as cytochrome *c* oxidase may reduce NO₂ to NO at low oxygen concentrations. This nitrite-dependent production was influenced by YHb, a flavohaemoglobin NO oxidoreductase (Castello *et al.*, 2006).

Fungi are able to produce NO during anaerobic conditions as a result of denitrification processes (Morozkina and Kurakov, 2007; Ye *et al.*, 1994). The mechanism consists of three consecutive reactions catalysed by three enzymes: NR, NiR and NoR. NiRs have been shown to produce NO as the major intermediate product. The fungi *Fusarium oxysporum*, *Cylindrocarpon tonkinense* and *Fellomyces fuzhouensis* (Abraham *et al.*, 1993; Kobayashi and Shoun, 1995; Kobayashi *et al.*, 1996; Uchimura *et al.*, 2002) have been found to possess a dissimilatory NiR, which catalyses the reduction of NO₂⁻ to NO associated with ATP synthesis in mitochondria. Moreover, nitrite-induced NO production from cultures of phytopathogenic fungi, i.e. *Pythium*, *Botrytis* and *Fusarium* spp., has been demonstrated using membrane-introduction mass spectrometry (MIMS)/restriction capillary inlet mass spectrometry (RIMS) (Conrath *et al.*, 2004).

NO SIGNALLING DURING PATHOGEN DEVELOPMENT

Several studies have investigated the developmental NO implications in fungal systems. Wang and Higgins (2005) reported the accumulation of NO in conidia and germinating hyphae of *Colletotrichum coccodes*, the intensity and location of which changed depending on the developmental stage of the fungus. Bio-imaging with the fluorochrome 3-amino,4-aminoethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA) showed the presence of NO in cells of the conidium, germ tubes and immature appressoria. However, the strongest NO-dependent fluorescence was observed in cells with reduced cytoplasm in the conidium with a mature appressorium, described as developmental stage E. An additional pharmacological approach demonstrated a regulatory role of NO during germination, as NO trapping accelerated germination and an excess of NO prevented this phenomenon. In addition, in the *B. cinerea* system, the production of NO was detected in all fungal developmental stages starting from spores up to mature mycelium (van Baarlen *et al.*, 2004; Turrión-Gómez and Benito, 2011). It is noteworthy that the NO-dependent fluorescence surrounding germinating spores and mycelium of *B. cinerea*, growing on both complete medium and *in planta*, revealed the diffusion of NO produced inside the fungal cells

(Floryszak-Wieczorek *et al.*, 2007; Turrión-Gómez and Benito, 2011). The observed spread of NO outside the fungal structures could have important physiological consequences in the establishment and progress of the disease.

NO is also involved in the control of both sexual and asexual spore development in fungi. As reported by Baidya *et al.* (2011), NO over-accumulation, caused by the absence of *fhbA* coding for the Fhb protein involved in NO decomposition, induced sexual development and decreased sterigmatocystin production in fungal cells. Simultaneously, supplementation of *A. nidulans* culture with exogenous NO promoted cleistothecial formation, accompanied by the expression of genes encoding the transcription factors, i.e. *nsdD* and *steA*, necessary for sexual development. Treatment of the *fhbA* mutant with NO positively affects mycotoxin production, suggesting that *fhbA* in *A. nidulans* could also influence secondary metabolism in fungi (Baidya *et al.*, 2011). In turn, in the basidiomycete *Flammulina velutipes*, NO was found to stimulate the formation of sexual fruiting bodies (Song *et al.*, 2000).

The development of sporangioophores in the zygomycete *P. blakesleeanus* (Maier *et al.*, 2001) and the photoconidiation process in *N. crassa* (Ninnemann and Maier, 1996) also seem to be under the influence of NO. Gong *et al.* (2007) indicated that a high level of NO is required for the initiation and development of conidiation in *C. minitans*. Bio-imaging revealed a strong NO signal in young pycnidia and in the undifferentiated tissue of pycnidial primordia; in turn, weak fluorescence signals were observed in growing hyphal tips or hyphae in which pycnidia or primordia did not develop (Gong *et al.*, 2007). Importantly, *Cryphonectria parasitica*, the plant pathogen causing chestnut blight, failed to produce pycnidia in response to both L-NAME and cGMP blocker (6-anilinoquinoline-5,8-quinone), suggesting that the NO-mediated signal for conidiation may be common to multiple fungal genera producing pycnidia (Gong *et al.*, 2007).

In the biotrophic fungus *Blumeria graminis* f.sp. *hordei*, Prats *et al.* (2008) detected a transient NO generation corresponding in time with appressorium maturation. In addition, application of either NO scavenger or a mammalian NOS inhibitor affected appressorial lobe formation (Prats *et al.*, 2008). NO has a regulatory effect on trap formation of nematode trapping fungi *Drechslerella stenobrocha* by acting contrary to abscisic acid (Xu *et al.*, 2010).

Further support for the signalling role of NO during fungal development has been provided by Vieira *et al.* (2009). An increased level of NO, correlating with the expression of guanylyl cyclase and cGMP phosphodiesterase genes, was detected during sporulation in the blastocladiomycete *Bl. emersonii*, an aquatic saprophytic lifestyle fungus. The pharmacological approach provided evidence that the Ca²⁺-NO-cGMP signalling pathway facilitated the control of zoospore biogenesis in the aquatic fungus (Vieira *et al.*, 2009). The presence of genes involved in the synthesis and/or degradation of cGMP was not shown only in *Bl. emersonii*; therefore, the NO-cGMP signalling pathway could

operate in other fungi as well (Vieira *et al.*, 2009). Interestingly, cyclic adenosine monophosphate (cAMP) is considered to be one of the major second messengers regulating morphogenesis and virulence in a wide variety of fungi, including plant pathogens, i.e. *Uromyces appendiculatus*, *Magnaporthe grisea*, *Colletotrichum* and *Fusarium* species (Priyatno *et al.*, 2012). cAMP has been demonstrated to be involved in mating, hyphal morphogenesis, infection structure formation, sclerotium formation, sporulation and the regulation of 'pathogenic' and 'saprophytic' spore germination, indicating that cAMP signalling is a key aspect of fungal phytopathogenesis (Barhoom and Sharon, 2004). As most of these processes are accompanied by NO generation, there is a possibility that the interaction between cAMP signalling and NO production exists in a similar manner as in the animal kingdom (Zhang and Hintze, 2001).

NO metabolism itself also plays a relevant role in the intimate association of plant growth-promoting rhizobacteria and roots to benefit crop plants (Molina-Favero *et al.*, 2008).

NO AND BACTERIAL STRESS RESPONSES

In the radiation-resistant bacterium *Deinococcus radiodurans*, bNOS is likely to be involved in the regioselective nitration of tryptophan at the 4-position by complex formation with tryptophanyl-tRNA synthetase (Buddha *et al.*, 2004). However, the role of 4-nitrotryptophan in the bacterium has not been identified; it appears to be implicated in the recovery from damage induced by UV radiation (Crane, 2008) and/or in the production of as yet unknown secondary metabolites (Sudhamsu and Crane, 2009).

NO AND FUNGAL STRESS RESPONSES

Zheng *et al.* (2009) found that exposure of the basidiomycete *Inonotus obliquus* to a fungal elicitor prepared from the plant-pathogenic *Alternaria alternata* led to enhanced NO production. The boosted NO production has been demonstrated to mediate the biosynthesis of polyphenols and to affect its metabolic profile. Moreover, higher cellular NO levels coincided with a greater accumulation of S-nitrosothiols (SNOs) and higher activity of enzymes controlling cellular levels of SNOs, i.e. S-nitrosogluthathione reductase (GSNOR) and thioredoxin reductase (TrxR), finally protecting *I. obliquus* cells from nitrosative stress (Zheng *et al.*, 2011). It is worth pointing out that S-nitrosylation is crucial for plant immunity, as it controls the activity and/or subcellular localization of the transcription factors non-expressor of pathogenesis-related genes 1 (NPR1), TGA1 and salicylic acid-binding protein 3 (SABP3) (Lindermayr *et al.*, 2010; Tada *et al.*, 2008; Wang *et al.*, 2009).

Another line of evidence has shown that NO is also engaged in abiotic stress responses in fungi. In yeast cells exposed to anoxia, Castello *et al.* (2006) demonstrated the activation of a signalling

pathway that involves a transient protein tyrosine nitration mediated by mitochondrially generated NO and associated with the induction of *CYC7*, a hypoxic yeast gene. Low doses of exogenous NO provided protection against oxidative stress in *Sa. cerevisiae* cells exposed to heavy metal, heat shock stress and high hydrostatic pressure (Chiang *et al.*, 2000; Domitrovic *et al.*, 2003). Furthermore, in mycelial cells of *Pleurotus eryngii* var. *tuoliensis*, exogenous NO applied as sodium nitroprusside (SNP) mitigated oxidative damage caused by heat stress (Kong *et al.*, 2012a). Finally, it must be stressed that endogenous NO is required for the synthesis of a nonreducing disaccharide, trehalose, during heat stress in *Pl. eryngii* var. *tuoliensis* (Kong *et al.*, 2012b).

It is well documented in higher eukaryotes that NO can influence programmed cell death (PCD) as both a pro- and anti-apoptotic factor. Almeida *et al.* (2007) also found that yeast cells undergo apoptosis-mediated NO signalling. Yeast apoptotic cell death, induced by H₂O₂ or age-associated factors, was accompanied by L-arginine-dependent NO synthesis and subsequent NO-mediated S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Almeida *et al.*, 2007). In plant cells, this key glycolytic enzyme undergoes S-nitrosylation, followed by translocation to the nucleus, interacting with DNA and proteins, such as the osmotic stress-activated protein kinase NtOSAK (Holtgreffe *et al.*, 2008; Wawer *et al.*, 2010). In turn, in mammalian cells, S-nitrosylated GAPDH, after translocation to the nucleus, triggers apoptosis (Hara *et al.*, 2005). It is worth pointing out that S-nitrosylation has been shown to modulate hypersensitive cell death during the plant response to pathogens through the regulation of NADPH oxidase (Yun *et al.*, 2011) or peroxiredoxin IIE (Romero-Puertas *et al.*, 2008).

NO AS A VIRULENCE FACTOR IN BACTERIA

The production of NO has significant implications in the virulence of bacterial pathogens. There is evidence that NO generated by these microorganisms may be responsible for the activation/deactivation of phytotoxins. NO synthesized via bNOS has been proven to nitrate the tryptophanyl moiety of taxtomin A, determining the phytotoxicity of this compound in *Streptomyces* spp. (Kers *et al.*, 2004; Wach *et al.*, 2005). Thus, the nitrated form of the toxin is essential for plant pathogenicity in most pathogenic streptomycetes (Loria *et al.*, 2008). The observed NO generation by *Streptomyces* strains was found to significantly exceed the level of NO needed for thaxtomin biosynthesis, and NO synthesis was localized strictly at the site of infection (Johnson *et al.*, 2008), suggesting a role for bNOS-derived NO also during host tissue colonization (Sudhamsu and Crane, 2009). Interestingly, nitration of the lipopeptide arylomycin generated by *Streptomyces* sp. Tü 6075 enhances its antibacterial activity (Schimana *et al.*, 2002), which may be significant in the infestation of new ecological niches by these bacteria.

Moreover, the pathogenic Gram-positive soil bacterium *Bacillus anthracis* uses its own NO as a key defence against the immune oxidative burst, thereby establishing bNOS as an essential virulence factor (Shatalin *et al.*, 2008). In this mechanism, the overproduction of endogenous NO rapidly protects pathogens themselves from immunological oxidative stress by a direct catalase activation and suppression of the harmful Fenton reaction. However, genes that encode bNOS are also present in the genomes of numerous non-pathogenic soil bacteria (Gusarov *et al.*, 2009).

NO has been shown to activate various antioxidant genes in *Escherichia coli* and *Ba. subtilis* to protect microbial cells against oxidative and nitrosative stress (Gusarov and Nudler, 2005). The transcription factors OxyR, SoxR, NorR and Fur in *E. coli* and ResDE in *Ba. subtilis* activate corresponding regulons on reaction with NO (Gusarov and Nudler, 2005). OxyR is a transcription factor that regulates anti-oxidant defences, and its activity is dependent on six Cys residues. The most important is Cys199, which is flanked by the S-nitrosylation motif, suggesting regulation by NO (Marshall *et al.*, 2000). As indicated by Mur *et al.* (2006), this key Cys residue and flanking S-nitrosylation motif are conserved in OxyR proteins in many plant pathogenic bacteria, including *Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Ralstonia solanacearum* and *Xylella fastidiosa*.

It is worth pointing out that NO-mediated pathogenesis has been found in the interaction between the human bronchial epithelial cell line 16HBE14o and the bacterium *Moraxella catarrhalis* (Mocca and Wang, 2012). NO generated by the human pathogen was able to induce host cell secretion of tumor necrosis factor alpha (TNF- α) and modulated the expression of apoptotic proteins, triggering, in consequence, host programmed cell death. Moreover, bacterium-derived NO blocked host cell division and resulted directly in the death of dividing cells by reducing the levels of an essential regulator of cell division (Mocca and Wang, 2012).

NO AS A VIRULENCE FACTOR IN FUNGI

Currently, we have no information as to whether and to what extent NO mediates fungal pathogenicity. However, there is evidence that pathogen-derived NO or NO produced in synergy with the host tissues constitutes an important element determining the success of the fungal infection. For example, elimination of NO produced by *M. oryzae* significantly reduced the level of infection in a compatible interaction (Samalova *et al.*, 2013). A programme of boosted NO formation was observed during various plant–*B. cinerea* interactions. In contact with susceptible pelargonium plants, *B. cinerea* itself gains the capacity to generate huge amounts of NO, which cause a massive nitrosative stress, facilitating grey mould development (Floryszak-Wieczorek *et al.*, 2007). van Baarlen *et al.* (2004) observed that *Arabidopsis* leaf inoculation with *B. cinerea* resulted in prominent NO accumulation by the fungus 5 days after inoculation. In turn, during the

tomato–*B. cinerea* interaction, NO production in the mycelium was detected as early as 15 h post-inoculation (hpi), reaching a maximum within the following 48 hpi. Interestingly, the expression of the NO scavenging flavohaemoglobin *Bcfhg1* declined shortly after germination during the saprophytic growth of *B. cinerea* and, as was speculated by Turrión-Gómez *et al.* (2010), such an over-accumulation of NO may be adaptive for a necrotroph *in planta* by creating more favourable conditions for the promotion of host colonization. It is also supported by the fact that *B. cinerea* endo-polygalacturonase 1 (BcPG1), involving cleavage of the linkages between D-galacturonic acid residues in homogalacturonan, is necessary for full virulence of the pathogen (ten Have *et al.*, 1998), and elicits host cell death and various defence responses, including NO synthesis (Kars *et al.*, 2005; Vandelle *et al.*, 2006). Taken together, plant tissues accelerate NO formation in both *B. cinerea*, most probably via the NO₂[−]-dependent pathway, and in the host. This creates a threshold of NO concentration, which may reduce the plant defence against the necrotroph and favour host colonization (Floryszak-Wieczorek *et al.*, 2007; Turrión-Gómez and Benito, 2011).

PATHOGEN-DERIVED NO IN THE CHALLENGE OF NITROSATIVE STRESS IN PLANTS

There is evidence that the NO level is sensibly regulated within microorganisms, as they cope with endogenously produced NO and, on the other hand, NO is used by the host immune system to counter pathogen challenge (Tillmann *et al.*, 2011). Briefly, colonization of the host tissues by pathogens often results in an overproduction of NO and NO-derived molecules, which create boosted and pathophysiological levels of reactive nitrogen species (RNS), defined as nitrosative stress. However, pathogen persistence to high levels of NO and NO-related molecules has also been associated with their immunosuppression by multiple strategies to detoxify them.

NITROSATIVE STRESS RESPONSE IN BACTERIA

Several enzymes responsible for resistance to nitrosative stress have been identified in bacteria (Missall *et al.*, 2004). The crucial detoxifying systems include flavorubredoxin nitric oxide reductase, an enzyme functioning under anaerobic and microaerophilic conditions, and flavohaemoglobin (hmp), operating physiologically as a denitrosylase under a wide range of O₂ concentrations. Other detoxifying activities include NoR, contributed by *nfrA*-encoded periplasmic cytochrome c NiR (Poock *et al.*, 2002), and GSNOR activity, associated with the *adhC*-encoded alcohol acetaldehyde dehydrogenase (Liu *et al.*, 2001). Moreover, the AhpC protein, a member of the peroxiredoxin family, has been proven to decompose OONO[−] to NO₂ (Bryk *et al.*, 2000).

NO-detoxifying gene expression in bacteria is mediated by specific NO sensors. In the model bacterium *E. coli*, two direct NO sensors have been recognized (Tucker *et al.*, 2008). One of them, NorR, senses NO directly through a mononuclear non-haem iron centre and responds by switching on the expression of the flavorubredoxin NorVW. In turn, the other protein, NsrR, has been shown to sense RNS to switch on a regulon of at least 60 genes, including the *hmp* coding NO dioxygenase (NOD) (Tucker *et al.*, 2008). Genome screening of several bacterial phytopathogens revealed the conservation of the NO-detoxifying NOD (*hmp*) gene and NorR (Mur *et al.*, 2006). Interestingly, deletion of the *hmpX* gene from a plant pathogenic bacterium *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) resulted in a significant increase in NO level in host tissues, wherein mutants were unable to infect the *Saintpaulia ionantha* plant (Boccarda *et al.*, 2005). Conversely, expression of *hmpX* into an incompatible strain of *Ps. syringae* suppressed hypersensitive cell death in soybean cell suspensions and the hypersensitive response (HR) patches in *Arabidopsis*, indicating that scavenging of NO by certain pathogens is required for successful infection. As suggested by Boccarda *et al.* (2005), *HmpX* was engaged in the attenuation of host HR during infection by intercepting host-derived NO for the execution of the hypersensitive cell death programme.

NITROSATIVE STRESS RESPONSE IN FUNGI

Like bacteria, fungi possess a central system to metabolize NO, i.e. the flavohaemoglobin (Fhb) NOD enzyme. NOD activity has been described in the yeast *Sa. cerevisiae* (Liu *et al.*, 2000), *Candida albicans* (Ullmann *et al.*, 2004), *Cryptococcus neoformans* (de Jesus-Berrios *et al.*, 2003), *A. oryzae* (Zhou *et al.*, 2011), *A. nidulans* (Zhou *et al.*, 2012) and *B. cinerea* (Turrión-Gómez *et al.*, 2010). Flavohaemoglobins exhibit a dioxygenase activity and contain three domains: a globin domain, a FAD-binding domain and a NAD(P)-binding domain. It was revealed by de Jesus-Berrios *et al.* (2003) that flavohaemoglobin denitrosylase contributes to *Cr. neoformans* pathogenesis. The human fungal pathogen lacking FHB1 flavohaemoglobin showed attenuated NO consumption concomitant with the inhibition of mutant cell growth by nitrosative challenge and displayed a virulence defect. Flavohaemoglobin-like proteins have also been identified in numerous plant fungal pathogens, including *Cladosporium fulvum*, *F. oxysporum* and *Gibberella zeae* (Boccarda *et al.*, 2005); however, the *in vivo* ability to detoxify NO via NOD activity has only been evidenced for *B. cinerea*. The generation of NO in *B. cinerea* has been well documented (van Baarlen *et al.*, 2004; Floryszak-Wieczorek *et al.*, 2007; Turrión-Gómez and Benito, 2011) and seems to be a dynamic process resulting from opposing mechanisms of NO production and degradation (Turrión-Gómez and Benito, 2011). Similar to the yeast (*Sa. cerevisiae*) model, the expression of the Fhb coding gene *Bcfhg1* in *B. cinerea* has been

shown to be developmentally regulated (Turrión-Gómez *et al.*, 2010), with maximum expression levels during the germination of conidia. In contrast with the human pathogens *Cr. neoformans* (de Jesus-Berrios *et al.*, 2003) and *Ca. albicans* (Ullmann *et al.*, 2004), deletion of *Bcfhg1* did not affect the pathogenicity of *B. cinerea*. The flavohaemoglobin-deficient mutants show no alteration in pathogenicity in relation to three different host species, i.e. *Solanum lycopersicum*, *Arabidopsis thaliana* and *Proteus vulgaris*. This result seems to suggest that the fungus Fhb is not engaged in exogenous NO decomposition derived from the host tissues challenged by the pathogen (Turrión-Gómez *et al.*, 2010). As, in some fungal species, Fhb coding genes are missing, there is a possibility that the protein function is either not essential for survival or can be taken over by other enzymatic or nonenzymatic systems. A specific fungal candidate could be a trehalose, which contributes to protection against RNS by stabilizing membranes and native proteins, as well as by suppressing the aggregation of denatured proteins in fungi (Doehleemann *et al.*, 2006). Moreover, NO was required for the regulation of trehalose accumulation during abiotic stress in *Pl. eryngii* var. *tuoliensis* (Kong *et al.*, 2012b), suggesting the existence of a positive feedback loop between NO and trehalose synthesis. It is worth pointing out that the yeast Yhb1 protein under hypoxic conditions could be translocated to the mitochondria, where it decomposes over-accumulated NO (Cassanova *et al.*, 2005). This provides evidence that flavohaemoglobins protect against nitrosative stress derived from both exogenous and endogenous sources.

To counteract nitrosative stress, yeasts also employ antioxidant mechanisms, including GSNOR, metalloporphyrins and trehalose (Tillmann *et al.*, 2011). So far, the mechanisms of NO sensing in fungi are poorly understood. However, Sarver and DeRisi (2005), in the *Sa. cerevisiae* yeast model, identified a positive regulator—the Cys₂His₂ zinc-finger factor Fzf1p—which is required for NO induction of *Yhb1* transcription. In turn, a zinc-finger protein CTA4 is essential for the induction of *Yhb1* transcription in *Ca. albicans* (Chiranand *et al.*, 2008). In the same model system, Cwt1p was found to act as a repressor of nitrosative stress responses involving the key NO-detoxifying enzyme Yhb1, as well as other NO-responsive transcripts (Sellam *et al.*, 2012). Importantly, *Ca. albicans* Δ cta4 null mutant resulted in attenuated virulence in the mouse model of systemic candidiasis, reinforcing the idea that robust nitrosative stress responses contribute to the pathogenicity of *Ca. albicans* (Chiranand *et al.*, 2008; Tillmann *et al.*, 2011).

CONCLUSIONS

Pathogens of different lifestyles have adopted sophisticated strategies to secure their survival and reproduction. The reviewed experimental evidence provides strong support for the concept of the functional role of NO in the offensive strategy of pathogens, as

Fig. 1 The mode of nitric oxide (NO) action in the pathogen unit (A) and the role of pathogen-derived NO during susceptible plant–pathogen interactions (B). NO generated by pathogenic microorganisms via the L-arginine and/or nitrite pathway (red lines) is directly or indirectly [via the guanosine 3',5'-monophosphate (cGMP) pathway] involved in various developmental processes, and may also be responsible for the activation of toxins and virulence factors. NO accumulation by the invading pathogen may induce S-nitrosylation of host membrane receptors and/or channels to effectively facilitate cellular entry. When the invader colonizes plant tissues, it destroys intracellular compartments of the host, which contributes to uncontrolled NO overproduction. Simultaneously, the host plant activates various defence responses, including its own programme of boosted NO production. Such huge amounts of NO in the susceptible genotype provoke nitrosative stress, leading to uncontrolled tyrosine nitration, facilitating tissue damage and colonization by the pathogen. Blue lines and areas, pathogen-derived NO; white areas outlined in green, plant-derived NO.

they synthesize NO and utilize NO production to their own benefit. In the physiological state or during host tissue colonization, pathogens are able to tightly regulate the balance between NO and other RNS, which could be a consequence of an adaptation mechanism to the presence of huge amounts of NO derived from internal and external sources. Importantly, the pathogen metabolic equipment to quench or reset the NO signal and counteract nitrosative stress not only plays a role as a modulator of the host immune response, but might also be implicated in virulence (Fig. 1). The biological roles of NO in multiple species of microorganism might be governed not only by the degree of pathogenicity, but also by the pathogen lifestyle.

Unfortunately, a complete picture of the strategies and mechanisms for the mode of NO action is not available for any of the plant pathogens studied to date. We have just begun to appreciate NO generation by plant pathogens, and many key questions remain to be answered, including the origin of pathogen-derived NO, its cellular targets, the NO degradation pathways and the mechanism of signal transduction. A knowledge of NO sensing

and signalling in model bacteria and human fungal pathogens could help us to select the appropriate research tools in order to reveal the fate of endogenous NO across multiple genera of plant pathogenic microorganisms. However, this cannot simply be performed by genome sequence comparisons, as many of these microorganisms lack obvious homologues of numerous genes involved in NO metabolism (Tillmann *et al.*, 2011). In this case, the application of proteomic and genomic tools for the detection of NO metabolic status in plant pathogens seems to be promising. Further research on necrotrophic and biotrophic plant pathogen systems is needed, as the pathogen lifestyle might determine not only the system responsible for the production and resetting of NO and the fate of pathogen-derived NO, but, above all, a full understanding of the role of NO during plant–pathogen interactions.

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