Nitric oxide mediates the fungal-elicitor-enhanced biosynthesis of antioxidant polyphenols in submerged cultures of *Inonotus obliquus*

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A fungal elicitor prepared from the cell debris of the plant-pathogenic ascomycete *Alternaria alternata* induces multiple responses by *Inonotus obliquus* cells, including an increase in generation of nitric oxide (NO), activity of phenylalanine ammonia lyase (PAL) and accumulation of total mycelial phenolic compounds (TMP), but does not trigger production of oxylipins or jasmonic acid (JA). The role of NO in TMP production was investigated via the effects of the NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO) and the nitric oxide synthase (NOS) inhibitor aminoguanidine (AG). TMP profiles were assayed using ¹H NMR spectroscopy combining multivariate pattern recognition strategies. Pretreatment of *I. obliquus* mycelia with cPITO or AG suppressed not only elicitor-enhanced NO generation and PAL activity, but also the elicitor-induced increase in TMP production. This TMP reduction by either a NO scavenger or a NOS inhibitor was reversed by exogenous addition of either a NO donor, sodium nitroprusside, or JA separately. NMR-based metabonomic analysis of TMP profiles showed that the induced TMP were hispidin analogues including inoscavins, phelligrins, davallialactone and methyldavallialactone, which possess high antioxidant activities. Thus, NO mediates an elicitor-induced increase in production of antioxidant polyphenols in *I. obliquus* via a signalling pathway independent of oxylipins or JA, a mechanism which differs from those in some higher plants.

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INTRODUCTION

The medicinal fungus *Inonotus obliquus* (Fr.) Pilat has been used as a folk medicine in Russia and Northern Europe for more than four centuries, where its effective use for treatment of various human malignant tumours and other diseases, in the absence of any unacceptable toxic side-effects, has been established (Zheng *et al.*, 2009a). Chemical investigations have revealed that *I. obliquus* produces a range of phenolic compounds, including hispidin analogues (Lee & Yun, 2007) and melanins (Babitskaia *et al.*, 2000). Of these, hispidin analogues are thought to be the bioactive constituents responsible for treating oxidative-stress-induced human diseases, including cancer, hypertension, neurodegenerative diseases and autoimmune diseases (Zheng *et al.*, 2009a). In nature, this fungus is

restricted to cold habitats (45° N–50° N latitude) and is found growing primarily on the trunks of *Betula* trees, forming sclerotia, the medically active fungal structures, after 10 to 15 years growth (Ham *et al.*, 2009). Its prominent bioactive metabolite synthesis has meant that demand for this fungus for pharmaceutical purposes has increased and sclerotia are no longer an adequate source of such compounds (Zheng *et al.*, 2009a).

In its natural habitats, *I. obliquus* is exposed to environmental stresses (Hoshino *et al.*, 1998; Zucconi *et al.*, 2002), including invasion by pathogenic microbes (Bolwell *et al.*, 2001). Strategies for surviving such conditions involve minimizing any stress-induced damage of lipids and DNA and attack by pathogenic microbes. Production of antioxidant hispidin analogues by *I. obliquus* is thought to be one such defence response (Zheng *et al.*, 2009b). Under normal culture conditions, this fungus produces small amounts of soluble melanins together with minor glycosylated flavonoids and hispidin analogues (Zheng *et al.*, 2009b). Imposing an oxidative stress by exposure to H₂O₂ resulted in production of insoluble melanins, yet

Abbreviations: AG, aminoguanidine; cPITO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; JA, jasmonic acid; NOS, nitric oxide synthase; PAL, phenylalanine ammonia lyase; PC, principal component; PCA, principal component analysis; RO, reverse osmosis; SNP, sodium nitroprusside; TMP, total mycelial phenolic compounds; TMS, tetramethylsilane.

hispidin analogues were only minor components of these (Zheng *et al.*, 2009b), and their immuno-stimulating effects were only about 50 % of those from the naturally grown fungus (Zheng *et al.*, 2008b). This implies that direct oxidative stress in submerged cultures of *I. obliquus* does not enhance accumulation of biologically active polyphenols.

In higher plants, cells respond to attack from pathogenic microbes by activating a wide variety of protective mechanisms designed to prevent their replication and colonization (Modolo *et al.*, 2002). Such defences include rapid localized cell death and accumulation of phytoalexins, which play an important role in many plant–pathogen incompatibility interactions (Modolo *et al.*, 2002). Applying elicitors from pathogenic fungi has been an effective strategy for improving the productivity of useful secondary metabolites in plant cell cultures (Roberts & Shuler, 1997). Such application activates several responses, including ion fluxes across the plasma membrane, synthesis of reactive oxygen species, and phosphorylation and dephosphorylation of proteins. These are widely reported putative components of signal transduction chain(s) leading to elicitor-induced defence responses, which include activation of defence genes, hypersensitive cell death and systemic acquired resistance (Baker & Orlandi, 1995). The molecular basis of these defence responses is believed to involve signal transductions by nitric oxide via a jasmonic acid-dependent signalling pathway and eventually biosynthesis of secondary metabolites (Xu *et al.*, 2005). However, relatively little is known about fungal-elicitor-induced increases in production of phenolic compounds and their mechanism of action. We showed earlier that adding cell wall debris from the plant-pathogenic fungus *Alternaria alternata* resulted in a three- to fourfold increase in total mycelial phenolic compounds (TMP) accumulation in *I. obliquus* (Zheng *et al.*, 2008a), which encouraged an investigation into fungal-elicitor-induced early events in this fungus and their role in the accumulation of phenolic compounds, in particular hispidin analogues.

Biosynthesis of hispidin analogues in *I. obliquus* involves the expression of enzymes in the phenylpropanoid pathway and a partitioning of precursors leading to the biosynthesis of hispidin (Zheng *et al.*, 2009b). Metabonomics-based approaches, capable of measuring the dynamic multiparametric responses of living systems to internal and external influences (Nicholson *et al.*, 1999), are claimed to evaluate comprehensively multiparametric metabolic responses to all pathophysiological stimuli and genetic modification (Gao *et al.*, 2008). A major technique in metabonomics, NMR spectroscopy, has the disadvantage of low detection limits, but possesses several advantages over HPLC/MS in that NMR measurements are non-destructive and non-selective, and it is feasible to acquire profiles of a comprehensive range of organic metabolites (Beckwith-Hall *et al.*, 2002; Li *et al.*, 2007). In this study, submerged cultures of *I. obliquus* were exposed to the fungal elicitor derived from *A. alternata* and relationships

between elicitor-induced early events, PAL activity and TMP production were analysed. The resultant phenolic profiles of mycelia exposed to the elicitor were also compared to those from cultures grown under normal physiological conditions using ^1H NMR spectroscopy, combining multivariate pattern recognition strategies.

METHODS

Fungal materials and preparation of inoculum in submerged cultures. *Inonotus obliquus* (Fr.) Pilat (KLBMP04005) was obtained from the fungal culture collection of the Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, China, and maintained on potato dextrose agar (PDA) (Shanghai Biotech). For preparing a standardized inoculum, the fungus was grown initially on PDA medium for 7 days and then transferred with a sterile self-designed cutter to a 500 ml conical flask containing 150 ml medium, consisting of glucose (2 %), peptone (0.35 %), yeast extract (2 %), KH_2PO_4 (0.01 %), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 %). Cultures were incubated with shaking on an orbital platform shaker (Shen Neng Bao Cai) at 26 °C and 140 r.p.m. for 4 days, and mycelium was then inoculated into 200 ml medium with the same composition in 500 ml conical flasks.

Elicitor preparation. The elicitor was prepared from a liquid culture of an isolate of the plant-pathogenic fungus *A. alternata* (purchased from the Fungal Collection Center, Institute of Microbiology, Chinese Academy of Sciences). Liquid cultures were initiated from cultures grown for 7 days on PDA, by inoculating blocks of agar (2 × 2 cm) into 500 ml conical flasks containing 150 ml potato dextrose liquid medium (Shanghai Biochemical). Cultures were incubated in darkness at 140 r.p.m. and 26 °C for 5 days. The mycelia were collected by filtration and washed three times with reverse osmosis (RO) water followed by disruption using ultrasonication (JY92-2D, Scientz) at room temperature for 100 min at 20 s intervals in RO water. The disrupted mycelia were filtered through a 0.45 µm filter; the filtrate was centrifuged three times (10 000 g for 10 min at room temperature) and the resultant cell debris (with diameters less than 0.45 µm) was dried by lyophilization and autoclaved for the following experiments.

Supplementation of submerged cultures with the elicitor and jasmonic acid. Fungal elicitor was added aseptically into media, 48 h after inoculation of *I. obliquus*, at final concentrations of 20, 40 and 60 µg l⁻¹. Jasmonic acid (JA) was also added to cultures 48 h after inoculation at a final concentration of 50 µg l⁻¹. Incubation of these supplemented cultures continued on an orbital platform shaker for another 12 days under conditions identical to those used in inoculum preparation.

Determination of mycelial biomass and total phenolic compounds. Mycelial biomass was determined by filtering culture broth using a pre-weighed GC filter paper, according to the procedures detailed previously (Zheng *et al.*, 2009a). For measuring the concentration of total phenolic compounds, mycelial samples were washed three times with RO water, and then extracted with anhydrous ethanol and ultrasonication (JY92-2D, Scientz) as described previously (Zheng *et al.*, 2009a). Total phenolic compounds were determined according to procedures described by Singleton & Rossi (1965) and expressed as gallic acid equivalents (GAE), using a standard curve generated with 0–80 mg gallic acid l⁻¹ (Sigma).

Determination of elicitor-induced generation of NO and oxylipins, and changes in NOS activity. Nitric oxide (NO) generation and nitric oxide synthase (NOS) activity were determined

using commercially available assay kits (Nanjing Jiangcheng Biotechnology Institute). For determining phenylalanine ammonia lyase (PAL) activities, washed mycelial pellets were homogenized by ultrasonication with 50 mM Tris/HCl buffer (pH 8.0) and enzyme activity was determined as detailed by Mori *et al.* (2001). Protein content was determined by the Coomassie blue-binding method (Bradford, 1976) using protein reagent (Bio-Rad) and BSA as standard. NO levels in culture broths are expressed as micromolar quantities, and NOS activity as units per mg protein, where one unit is defined as the capacity to produce 1 nmol NO in 1 min. For measuring elicitor-induced biosynthesis of oxylipins, 10 mg lyophilized mycelium powder plus 0.5 mg oleic acid (internal standard, locally purchased with purity more than 99%) was extracted ultrasonically with 1 ml n-hexane (locally purchased with HPLC chromatographic purity) in an ice bath (to prevent solvent evaporation) for 1 h, followed by centrifugation at 4800 g for 10 min. The resultant supernatant was used for GC/MS (Hewlett Packard, 5890) and analysed by the procedure detailed by Assaf *et al.* (1995). The amounts of precursor linoleic acid and end product oxylipins present were expressed as a percentage relative to the total volatile constituents given by the workstation of the HP 5890.

Test of elicitors and inhibitors and their relevance to PAL activity and production of phenolic compounds. For experiments with inhibitors of the elicitor-induced increase in the production of phenolic compounds by *I. obliquus*, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO, Sigma), the NOS inhibitor aminoguanidine (AG, Sigma) and the NO donor sodium nitroprusside (SNP, Sigma) were dissolved in RO water, filtered through 0.22 µm sterile Acrodisc syringe filters (PALL) and added to 2-day-old mycelia cultures 20 min before addition of the elicitor (final concentration, 40 µg l⁻¹) in the following patterns: elicitor + AG (0.1 mM final concentration); elicitor + AG + SNP (0.02 mM final concentration); elicitor + AG + SNP + cPITO (0.5 mM final concentration). The mycelia were harvested every 2 h for determination of PAL activity according to the methods described previously (Zhao *et al.*, 2009), and every 2 days for measuring total phenolic compounds using the method described by Singleton & Rossi (1965). To further elucidate the signalling pathway leading to the defence responses in *I. obliquus*, JA was also added separately to cultures at a final concentration of 50 µM. The resultant PAL activities and accumulation of phenolic compounds were assayed as above. Controls received equivalent volumes of appropriate solvent.

¹H NMR spectroscopy measurements and signal assignments. Ethanol extracts of mycelia taken at day 13 from submerged cultures, with or without elicitor supplementation, were lyophilized and dissolved in 600 µl deuterated DMSO in an NMR tube in preparation for ¹H NMR measurements. Ten microlitres of tetramethylsilane (TMS) were added as an internal standard. ¹H NMR spectra were recorded at 25 °C at a ¹H frequency of 400.13 MHz on a Bruker Avance NMR spectrometer (Bruker BioSpin) equipped with a standard 5 mm BBO probe, using a standard one-dimension spectral acquisition procedure. A total of 128 transients of 8K data points spanning a spectral width of 10.00 p.p.m. was collected. A 0.5 Hz exponential line-broadening function was applied to the FID (free induction decay) prior to the FT (Fourier transform). All spectra were referenced to the TMS signal at 0 p.p.m. Signal assignments were conducted by referencing the proton signals of standards and the data reported previously (for references, see Results). The standards included hispidin analogues, benzoic acid derivatives and flavonoids. Hispidin analogues (with purities of more than 96%) were isolated from naturally grown *I. obliquus* according to the procedure described by Zheng *et al.* (2009c). These consisted of inoscavins A, B, C and D, phelligridins C, D, E and J, davallialactone and methyldavallialactone. The standards of benzoic acid derivatives and flavonoids and their sources were the same as listed previously (Zheng *et al.*, 2009b).

Data reduction and pattern recognition. The proton resonances of phenolic compounds produced by *I. obliquus* in ¹H NMR spectra are present predominantly in downfield, with chemical shifts (δ) of more than 4.20 p.p.m. (Lee & Yun, 2006). Thus ¹H NMR spectra (δ 9.00–4.20 p.p.m.) were automatically data-reduced to 120 integral segments of equal length (δ 0.04 p.p.m.) using Mestrec 4.86 (Mestrelab Research), with each segment consisting of the integral of the NMR region to which it was associated. The data were normalized to total spectral area and centred scaling was applied before pattern recognition analyses. Principal component analysis (PCA) was performed using a mean-centred approach with SIMCA P-11 (Umetrics) software. Spectral filters were applied to remove any unrelated components and partial least-squares discriminative analysis (PLS-DA) was used for pattern recognition. Data were displayed using the principal component (PC) score and loading plots.

Measurements of free radical scavenging capacities. The scavenging potential of superoxide radicals was analysed with a hypoxanthine/xanthine oxidase-generating system coupled to NBT (nitro blue tetrazolium) reduction, as detailed by Zheng *et al.* (2009b). Potential for scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) was conducted according to the protocol of Shyu & Hwang (2002) and capacity for scavenging hydroxyl radicals was determined following the procedure described by Zheng *et al.* (2009a). The scavenging capacities were represented as the percentage inhibition of the tested free radicals by 1 mg ethanol extracts.

Statistics. All the experiments consisted of ten independent repeats. Results from representative experiments are expressed as mean ± SD. Data from all experiments were analysed by *t*-test (SPSS 11.0). The assumptions of analysis of variance were considered to be statistically significant at *P* < 0.05.

RESULTS

Elicitor-enhanced production of total phenolic compounds

Fig. 1 shows the elicitor-induced increase in TMP accumulation in *I. obliquus*. Under normal physiological conditions, the highest TMP level occurred after day 13 at a level of 31.45 mg g⁻¹. Addition of fungal elicitor at several concentrations resulted in a marked enhancement in TMP production. For example, addition at a concentration of 40 µg l⁻¹ led to an increase in TMP level to 70.56 mg g⁻¹ (*P* < 0.05) (Fig. 1a). No difference in the accumulation of mycelial biomass in the control cultures and those with elicitor addition was recorded, except that slightly lower mycelial biomass production was seen in cultures exposed to 20 µg elicitor l⁻¹ (Fig. 1b).

Generation of NO and oxylipins

As indicated in Fig. 2, NO generation increased 4 h after elicitor exposure and the concentration in the medium reached a maximum of 483.97 µM after 12 h, representing about a four- to fivefold increase above the control (Fig. 2a). The activities of NOS also increased after elicitor addition, which is consistent with the elicitor upregulating NOS activity in the *I. obliquus* mycelia (Fig. 2b). The data also showed that the elicitor and JA did not induce the

Fig. 1. Effects of fungal elicitor on accumulation of mycelial phenolic compounds (a) and mycelial biomass (b). Fungal elicitor was added 3 days after inoculation at final concentrations of 20 $\mu\text{g l}^{-1}$, 40 $\mu\text{g l}^{-1}$ and 60 $\mu\text{g l}^{-1}$. Results are the mean of ten independent experiments and error bars in (a) indicate standard deviation (not shown when smaller than symbols).

biosynthesis of oxylipins but increased the accumulation of linoleic acid (Fig. 2c).

Dependence of the elicitor-induced increase in PAL activity and TMP accumulation on NO generation

The above results indicated that NO generation was an early response to the fungal elicitor in *I. obliquus* cells. To investigate its possible involvement in the elicitor-induced increases in production of phenolic compounds, we determined the effects of a NO scavenger and a NOS inhibitor on elicitor-enhanced PAL activity and TMP production. As shown in Fig. 3, PAL activity was inhibited by adding the NO-specific scavenger cPITO and the NOS inhibitor AG (Fig. 3a). The reduction in TMP coincided with the inhibition of NO generation (Fig. 3b), suggesting that NO generation is the upstream signalling event essential for elicitor-enhanced TMP production by *I. obliquus*. This proposal gains further support from the restoration of elicitor-enhanced TMP accumulation after the addition of SNP to AG- and cPITO-supplemented cultures (Fig. 3b). In order to further elucidate the

signalling pathway leading to the defence responses in *I. obliquus*, the fungus was also exposed to JA at a final concentration of 50 μM . JA addition also enhanced PAL activity (Fig. 3c) and the subsequent increase of TMP (Fig. 3d).

Classes of phenolic compounds induced by the fungal elicitor

Elicitor-enhanced biosynthesis of polyphenols was evaluated by NMR-based metabolomic analysis using multivariate pattern recognition strategies. Ethanol extracts (10 ml) of mycelia grown in control and elicitor-supplemented medium (40 $\mu\text{g l}^{-1}$), taken at day 13, were lyophilized for ¹H NMR measurements. In the ¹H NMR spectra, a broadened singlet between 7.04 and 7.10 p.p.m. (Fig. 4 a, b) represents the typical proton resonance collectively contributed by H-9 in hispidin analogues (Jung *et al.*, 2008; Kim *et al.*, 1999; Kojima *et al.*, 2008; Lee *et al.*, 2006; Lee & Yun, 2006; Lee *et al.*, 2007; Mo *et al.*, 2004; Wang *et al.*, 2007). In the spectra of extracts from mycelia exposed to elicitor (Fig. 4 b), downfield singlet resonances between 7.85 p.p.m. and 8.51 p.p.m. suggest the presence of phelligidins C, D, E (Mo *et al.*, 2004), G (Kojima *et al.*, 2008), J and I (Wang *et al.*, 2007). The typical doublets with a coupling constant of 13.2 Hz at 5.81 and 5.93 p.p.m. (H-5') are consistent with the presence of davallialactone and methyldavallialactone (Lee & Yun, 2006). In addition, the two broadened singlets at 6.84 p.p.m. and 7.51 p.p.m. result from joint contributions of inoscavins B and C, and methyl inoscavin C (Kim *et al.*, 1999; Lee *et al.*, 2006; Lee & Yun, 2006). The proton resonances by benzoic acid derivatives and flavonoids were not obvious in the extracts from either control or elicitor-treated mycelia.

Visual comparison of these ¹H NMR spectra allows a qualitative recognition of marked shifts in resonances, particularly in the range between δ 9.00 and 7.60 p.p.m., and δ 5.90 and 5.40 p.p.m. in the patterns of extracts of mycelia grown in the control and elicitor-supplemented medium (Fig. 4a, b). For quantitative differentiation based on pattern recognition, chemometric methods including PCA were employed to minimize any instability that might have originated from variations in ethanol extract concentrations and inter-sample differences. Standard normal variate transformation (SNV) was employed as a spectral filter to remove any unrelated components, and partial least-squares discriminative analysis (PLS-DA) was performed on the normalized ¹H NMR datasets, unitizing mean-centred and scaled data. Substantial differences in the profiles of phenolic compounds were observed between the control mycelia and those exposed to the elicitor, and their dissimilarities are shown in the score plots drawn by SIMCA-P on Hotelling T2 (Fig. 4c). Two components were calculated and the model corresponding to the first PC accounted for 60.37% of the total variance. The region of spectra contributing most to this separation is indicated in the corresponding PC loading plot (Fig. 4d). Thus, the data

Fig. 2. Effects of fungal elicitor on NO generation (a), NOS activity (b) and production of oxylipins (c). Fungal elicitor was added 3 days after inoculation at a final concentration of $40 \mu\text{g l}^{-1}$ and mycelia were taken at intervals of 4 h for assaying NO and oxylipin contents and NOS activity, respectively. Results are the mean of ten independent experiments and error bars indicate standard deviation.

Fig. 3. (a, b) Effects of NO scavenger and NOS inhibitor on elicitor-induced increases in PAL activity (a) and accumulation of TMP (b). (c, d) JA-induced increases in PAL activity (c) and accumulation of TMP (d). cPITO and AG, respectively, were added to 2-day-old mycelial cultures 20 min before addition of the elicitor at $40 \mu\text{g l}^{-1}$. JA was added to 2-day-old cultures at a final concentration of $50 \mu\text{mol l}^{-1}$. The mycelia were harvested every 2 h for determination of PAL activity and every 2 days for measuring TMP. Results are the mean of ten independent experiments and error bars indicate the standard deviation (not shown when smaller than symbols). GAE, gallic acid equivalents.

Fig. 4. Representative ¹H NMR spectra of ethanol extracts from mycelia grown in control medium (a) and elicitor-added medium (b), and dissimilarities in phenolic profiles in score (c) and loading plots (d). Score and loading plots were obtained by a mean-centred approach with SIMCA P-11 (Umetrics) software. The data were analysed using standard normal variate transformation (SNV) as spectral filter combined with least-squares discriminative analysis (PLS-DA). C, control; E, elicitor.

show that the elicitor primarily induced the biosynthesis of hispidin analogues, including phelligradins D, G and J, inoscavins B, C and D, methyl inoscavin C, davallialactone and methyl davallialactone.

Effects of elicitor on antioxidant activities of ethanol extracts

Ethanol extracts of mycelia grown in the control and elicitor-supplemented medium all possessed potentials for scavenging DPPH, superoxide anion and hydroxyl radicals, with increasing capacities for scavenging DPPH reflecting increased incubation times. Adding elicitor at a concentration of 40 µg l⁻¹ resulted in higher antioxidant activities compared to those in the control medium and those containing elicitor at concentrations of 20 and 60 µg l⁻¹ after day 11 ($P < 0.05$) (Fig. 5a). Similar trends for the ability to scavenge superoxide anion and hydroxyl radicals were also determined, where adding elicitor at 40 µg l⁻¹ also triggered increases in antioxidant activities after day 11 ($P < 0.05$) (Fig. 5b, c).

DISCUSSION

The data presented here show that an elicitor prepared from the cell walls of the plant-pathogenic fungus *A. alternata* enhances NO generation, PAL activity and TMP production in mycelia of *I. obliquus* grown in submerged

cultures. The TMP increase was blocked by a NO scavenger and a NOS inhibitor, implying that NO generation is essential. Addition of the elicitor to cultures of this fungus did not trigger the biosynthesis of oxylipins or JA, which implies that the elicitor-induced TMP increase is mediated by NO in the signalling pathway independent of these compounds, and instead involves hispidin analogues, including inoscavins, phelligradins, davallialactone and methyl davallialactone. Thus, it appears that NO mediates an elicitor-induced biosynthesis of hispidin analogues through a signalling pathway independent of oxylipins.

In basidiomycota, hispidin is thought to be synthesized either from phenylalanine, via cinnamyl derivatives (phenylpropanoid pathway) combined with acetate, from malonate through the polyketide pathway (Lee & Yun, 2007), or from the condensation of 4-hydroxy-6-methyl-2-pyrone, which is formed by the reaction of three molecules of acetyl-S-CoA and one molecule of 3,4-dihydroxybenzoyl-S-CoA (Mo *et al.*, 2004). In our study, pretreatment of mycelia with a NO scavenger or a NOS inhibitor not only suppressed this increased NO production, but also inhibited any increase of PAL activity and subsequent accumulation of hispidin analogues. This suggests that hispidin in *I. obliquus* is synthesized via the phenylpropanoid pathway.

Oxylipins are widely found in many species of ascomycota and some species of basidiomycota (Tsitsigiannis & Keller, 2007), where they play important roles in asexual and

Fig. 5. Effects of fungal elicitor on scavenging of DPPH (a), superoxide anion (b) and hydroxyl radical (c) of ethanol extracts from mycelia of *I. obliquus*. The scavenging capacities are represented as percentage inhibition of the tested free radicals by 1 mg ethanol extracts. Results are the mean of ten independent experiments and error bars indicate the standard deviation (not shown when smaller than symbols).

sexual development (Noverr *et al.*, 2003). They are formed after enzymic cleavage of linoleic acid (Combet *et al.*, 2006). Our results confirmed that oxylipins are not produced in mycelia grown in either control or elicitor-supplemented medium, but JA addition resulted in an enhanced expression of PAL (Fig. 3c) and TMP accumulation (Fig. 3d), which agrees with our previous data (Zhao *et al.*, 2009). This implies that enhanced biosynthesis of phenolic compounds in *I. obliquus* cells can be mediated by several signalling pathways, where exogenous or endogenous signal molecules such as NO and JA might enhance the expression of defence-related genes leading to the accumulation of phenolic compounds independently.

In higher plants, NO mediates biosynthesis of antimicrobial flavonoids in cells of *Glycine max* when exposed to attack by the fungus *Diaporthe phaseolorum* f. sp. (Modolo *et al.*, 2002), and induces a systemic resistance in tobacco by a salicylic acid (SA)-dependent signalling pathway (Song & Goodman, 2001). Similar work also showed that adding cell debris of *Aspergillus niger* to cell suspension cultures of *Hypericum perforatum* triggered NO biosynthesis that mediated production of the phenolic compound hypericin via a JA-dependent signalling pathway (Xu *et al.*, 2005). Thus NO can induce biosynthesis of phytoalexins in cells of these plants via a JA- or SA-dependent signalling pathway, which seems to differ fundamentally from the elicitor-induced defence responses in *I. obliquus*.

NO is a ubiquitous small molecular messenger whose role as a signal transducer in plants (Zhao *et al.*, 2005) is becoming clearer. Under physiological conditions, NO is barely detectable in cell suspension cultures like those of *Hypericum perforatum* (Xu *et al.*, 2005), but could be detected in submerged cultures of *I. obliquus* grown under normal physiological conditions (Fig. 2a). In plants, NO can be synthesized either non-enzymically via light-mediated conversion of NO_2 by carotenoids, or enzymically from NO_2 by NADPH nitrate reductase (Foissner *et al.*, 2000; Neill *et al.*, 2002), and possibly from L-arginine by a mammalian-type NOS in the presence of O_2 (Durner & Klessig, 1999; Neill *et al.*, 2002). In *I. obliquus*, NOS activity remained detectable in mycelia grown in control medium but increased to three to four times the level of the control in cultures exposed to the elicitor (Fig. 2b). This might suggest that NOS-like enzymes in *I. obliquus* are expressed in physiological conditions differing from those observed in plants.

It is clear that NO acts as a key signal in plant resistance to pathogens by activating the expression of several genes leading to increases in the activities of PAL and chalcone synthase and the biosynthesis of antimicrobial flavonoids (Romero-Puertas & Delledonne, 2003), where phenylalanine, the precursor of phenylpropanoid metabolism, is directed primarily to flavonoid synthesis. In *I. obliquus*, elicitor-enhanced NO production also results in a marked increase in PAL activity but this drives the metabolic fate of phenylalanine towards the biosynthesis of hispidin analogues.

It is believed that NO production in plants induced by pathogen attack initiates several defence responses including cell death, cellular damage and activation of defence genes. However, its excessive production may result in the formation of ONOO^- , a highly toxic radical, in the presence of superoxide anion (Durner & Klessig, 1999), and this may cause serious harm to cells. In our study, addition of elicitor at 60 $\mu\text{g l}^{-1}$ also resulted in smaller TMP increases than those seen in medium containing 40 $\mu\text{g l}^{-1}$ elicitor (Fig. 1a). Thus, the reduced TMP production probably reflected their increased consumption in scavenging free radicals, including the toxic ONOO^- , which is also supported by *in vitro* antioxidant activity

data, where ethanol extracts of mycelia exposed to the elicitor at $60 \mu\text{g l}^{-1}$ showed less potential for scavenging free radicals than those exposed to $40 \mu\text{g l}^{-1}$ (Fig. 5).

It seems that phenolic metabolism and biosynthesis of hispidin analogues in *I. obliquus* are affected by a wide range of environmental factors imposing oxidative stress on this fungus (Zheng *et al.*, 2009b). However, production of hispidin analogues was not enhanced after addition of H_2O_2 to the cultures (Zheng *et al.*, 2009a). Here, presence of elicitor in the medium resulted in both an enhanced accumulation of hispidin analogues and upregulation of antioxidant activities, which implies that it may act to modify phenolic metabolism in *I. obliquus* under laboratory conditions.

This study suggests that NO mediates elicitor-induced increase in PAL activity and subsequent biosynthesis of hispidin analogues in submerged cultures of *I. obliquus* using a signalling pathway independent of oxylipins or JA. Further work is needed to determine which other molecular messengers might be involved in this signalling pathway. Nevertheless, this study does reveal possible mechanisms of interactions between different fungal species as well as providing insights into phenolic metabolism that might be modified deliberately, and which may assist in efforts to obtain pharmaceutically active polyphenols from scaled-up cultures of *I. obliquus*.

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