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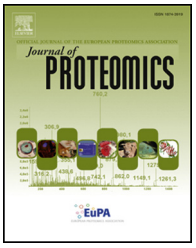

Mechanism of *Penicillium expansum* in response to exogenous nitric oxide based on proteomics analysis

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ABSTRACT

Penicillium expansum is an important fungal pathogen, which causes blue mold rot in various fruits and produces a mycotoxin (patulin) with potential damage to public health. Here, we found that nitric oxide (NO) donor could significantly inhibit germinability of *P. expansum* spores, resulting in lower virulence to apple fruit. Based on two dimension electrophoresis (2-DE) and mass spectrometry (MS) analysis, we identified ten differentially expressed proteins in response to exogenous NO in *P. expansum*. Among of them, five proteins, such as glutamine synthetase (GS), amidohydrolase, nitrilases, nitric oxide dioxygenase (NOD) and heat shock protein 70, were up-regulated. Others including tetratricopeptide repeat domain, UDP-N-acetylglucosamine pyrophosphorylase, enolase (Eno), heat shock protein 60 and K homology RNA-binding domain were down-regulated. The expression of three genes associated with the identified proteins (GS, NOD, and Eno) was evaluated at the mRNA level by RT-PCR. Our results provide the novel evidence for understanding the mechanism, by which NO regulates growth of *P. expansum* and its virulence.

Biological significance

Crop diseases caused by fungal pathogens lead to huge economic losses every year in the world. Application of chemical fungicides to control diseases brings the concern about food and environmental safety. Screening new antimicrobial compounds and exploring involved mechanisms have great significance to development of new disease management strategies. Nitric oxide (NO), as an important intracellular signaling molecule, has been proved to be involved in many physiological processes and defense responses during plant–pathogen interactions. In this study, we firstly found that NO at high concentration could distinctly delay spore germination and significantly reduce virulence of *P. expansum* to fruit host, identified some important proteins in response to NO stress and characterized the functions of these proteins. These results provide novel evidence for understanding the mechanism of NO regulating virulence of the fungal pathogen, but are beneficial for screening new targets of antifungal compounds.

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1. Introduction

Penicillium expansum is one of the most destructive fungal pathogens, and causes blue mold rot in various fresh fruits, resulting in huge economic losses in the world [1–3]. In addition, the pathogen can produce patulin, which is carcinogenic mycotoxin and damages human health [4]. Application of chemical fungicides to control *P. expansum* usually leads to the appearance of resistant strains and brings the concern about food and environmental safety. Thus, other strategies, including biological control with antagonistic microorganisms [1,5], applications of bioactive compounds [6,7] and exogenous plant regulators [8,9] and chemical compounds [3,10], have been widely used to control diseases caused by fungal pathogens in various fruits. These approaches have become new technologies for control of diseases instead of chemical fungicides.

Nitric oxide (NO) is an important intracellular signaling molecule involved in many physiological processes, and has been found in organisms throughout the phylogenetic scale, from fungi to mammals [11,12]. In recent years, some experiments demonstrated that NO treatment could extend shelf life of harvested horticultural crops [13–15], and proved the mechanisms by which NO possibly antagonize ethylene, salicylic acid, jasmonic acid, and other growth regulators [16,17]. Moreover, many reports have suggested that NO is involved in defense responses during plant–pathogen interactions [18] because NO can induce hypersensitive response, phytoalexin accumulation, and the onset of systemic acquired resistance [12,19]. Furthermore, Wang and Higgins [20] found that spore germination of *Colletotrichum coccodes* was delayed when the pathogen was incubated with a solution of sodium nitroprusside (SNP, NO-donor compound). Ghaffari et al. [21] pointed out that NO was toxic against *Candida albicans* and methicillin-resistant *Staphylococcus aureus*. Lazar et al. [22] observed that the growth of *Aspergillus niger*, *Monilinia fructicola*, and *Penicillium italicum* was suppressed after short-term exposure to a low concentration of NO. Hong et al. [23] demonstrated that both H_2O_2 and NO had antibacterial activity to *Ralstonia solanacearum* in a dose-dependent manner in vitro. Schairer et al. [24] proved that NO was a direct potent antimicrobial agent effectively against a range of microorganisms, including both bacteria and fungi. The antimicrobial properties of NO may be elicited by direct modification of biomacromolecules or by formation of reactive nitrogen species (RNS) via reaction with oxygen (O_2) or superoxide (O^{2-}) [25]. Additionally, NO, with its lone radical on the nitrogen atom, is implicated in a number of secondary mechanisms of toxicity, including catalase inhibition (resulting in hydrogen peroxide toxicity), Fe–S center iron liberation, and the formation of dinitosyl-iron complexes [26].

However, the mechanisms by which NO inhibits virulence of plant fungal pathogens are still unknown. We previously reported that NO released from SNP could significantly suppress spore germination of *P. expansum*, and found that there were lower level of ATP and higher level of reactive oxygen species (ROS) and carbonylated proteins in SNP-treated spores [2]. Since proteomics approach has been successfully used to explore the mechanisms of fungal pathogens in response to various stresses [27–29]. In this study, we investigated the mode of action of NO regulating spore germination of *P. expansum* based on proteomics approach. Further, the expressions of genes related to four important differentially expressed proteins were also evaluated by RT-PCR analysis. These results provide novel insights into exploring the mechanisms of NO regulating virulence of fungal pathogen.

2. Materials and methods

2.1. Fungal strain and antimicrobial effect assays

P. expansum link (CGMCC3.3703) was used in this study and cultured at 25 °C on potato dextrose agar (PDA) plates. Conidial suspension was prepared by flooding 10-day-old sporulating cultures with sterile distilled water, and filtered with four layers of cheese-clothes. A final concentration of 1×10^6 spores/ml in 100 ml potato dextrose broths (PDB) supplemented with 0 mmol/l or 6 mmol/l SNP was incubated at 25 °C on a rotary shaker at 200 rpm. During the incubation of 6 to 10 h, the germination rate per hour of about 100 spores in each treatment was microscopically determined. Then, 60 spores per treatment were assayed for germ tube length at 11, 13 and 15 h incubation, respectively. Each treatment was replicated three times and the experiment was repeated twice. After 6 h and 9 h incubation, these spores were directly sampled for microscopy or collected by centrifugation and used for subsequent experiments.

Antimicrobial effect assays in vivo were carried out in an apple fruit (*Malus domestica* Borkh. cv. Red Fuji). Fruits were washed in a 2% (v/v) sodium hypochlorite solution for 2 min, rinsed with tap water, and air-dried prior to use. Then fruits were wounded (4 mm deep, 3 mm wide) at the equator of each fruit using sterile nail and inoculated with 10 μl of the spore suspension at 1×10^4 spores/ml. Before inoculation, the spores were cultured in PDB, containing 0 or 6 mmol/l SNP, and shook on a rotary shaker at 200 rpm for 6 h at 25 °C. All fruits were put into plastic boxes with plastic film to maintain a high relative humidity (95%), and stored at 20 °C. Disease incidences and lesion diameters were investigated daily. There were 10 fruits in each treatment with three replicates and the experiment was repeated twice.

2.2. TEM analysis

The spores were incubated in PDB containing 0 or 6 mmol/l SNP for 6 h, and fixed with 2.5% formaldehyde and 2% paraformaldehyde in 0.1 mol/l sodium cacodylate buffer (SCB), pH 7.2 overnight, then centrifuged (16,000 *g* for 5 min at 4 °C). Gels of 1 to 2 mm^3 were prepared by adding 3% low gelling temperature agarose in SCB to the pellet. After thorough rinsing with 0.1 mol/l SCB, the gels were post-fixed with 1% osmium tetroxide in 0.1 mol/l SCB at room temperature for 4 h, and dehydrated with 15 min stages in a graded acetone series. The samples were embedded in Spurr resin. Ultrathin sections were obtained using a diamond knife and stained by soaking in 2% uranyl acetate for 15 min, and post-stained in lead citrate for 1 min. The sections were analyzed using a JEOL 1230 transmission electron microscope (Japan) at 80 kV.

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