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Original article

The role of nitric oxide in basal and induced resistance in relation with hydrogen peroxide and antioxidant enzymes

Vahid Keshavarz-Tohid[a], Parissa Taheri[a,*], Seyed Mohsen Taghavi[b], Saeed Tarighi[a]

[a] Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Plant Protection, Faculty of Agriculture, Shiraz University, Shiraz, Iran

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ABSTRACT

Nitric oxide (NO) is one of the main signal molecules, which is involved in plant growth and development and can change regular physiological activity in biotic and abiotic stresses. In this study, the role of NO in induced resistance with *Pseudomonas fluorescent* (CHA0) and basal resistance against *Rhizoctonia solani* in bean plant was investigated. Our results revealed that *P. fluorescent* and *R. solani* can increase NO production at 6 h post inoculation (hpi). Also, using the NO donor S-nitroso-*N*-acetyl D-penicillamine (SNAP) led to increase NO and bean plant resistance against *R. solani*. Utilizing the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethy-limidazoline-1-oxyl-3-oxide (cPTIO), not only decreased basal resistance but also reduced induced resistance. In continue, the activity of antioxidant enzymes was studied in the former treatments. SNAP, CHA0 and *R. solani* increased the activity of peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX) at 6, 12 and 24 h post inoculation (hpi). In contrast, using cPTIO and *R. solani* simultaneously (cPTIO + R) showed reduction in activity of POX and APX at 6 hpi. The cPTIO + R treatment increased POX, APX and CAT activity at 12 and 24 hpi. Hydrogen peroxide (H_2O_2) monitoring in the leaf discs clarified that SNAP can increase H_2O_2 production like CHA0 and *R. solani*. On the other hand, SNAP increased the resistance level of leaf discs against *R. solani*. Treating the leaf discs with cPTIO led to decrease resistance against the pathogen. These leaf discs showed reduction in H_2O_2 production at 6 hpi and suddenly enhanced H_2O_2 generation was observed at 24 hpi. This study showed that CHA0 can increase NO level in bean plants. NO induced H_2O_2 generation and regulated redox state of the host plant. This interaction resulted in significant defense against the pathogen.

1. Introduction

Nitric oxide (NO) is produced in many organisms such as animals, algae, yeasts, bacteria and plants. The role of NO in plant physiology was known in 1998 (Delledonne et al., 1998) and after that, diverse aspects of this molecule have been investigated for determining its capabilities.

These days, it is clarified that NO has an important role in plant development and resistance against biotic and abiotic stresses. Recognition of pathogen-associated molecular pattern (PAMP) and microbe-associated molecular pattern (MAMP) via pattern recognition receptors (PRRs) of plants leads to increased levels of NO. Several pathways are involved in NO production belonging to two major categories, known as oxidative and reductive pathways (Gupta et al., 2011; Mur et al., 2013). Generation of NO occurs in different organelles of plant cells. Involvement of L-arginine in NO biosynthesis by the activity of nitric oxide synthase (NOS)-like enzyme and polyamines oxidation, hydroxylamine, and nitrite-dependent NO production (via the activity of nitrate reductase; NR) is observed in the cytosol, chloroplast and peroxisomes. Another organelle that generates NO based on reduction is mitochondria, which produces this gaseous molecule in the lack of oxygen to protect O_2 (Gupta et al., 2009). NO production in mitochondria is dependent on nitrite reduction (Mur et al., 2013). Also, NO production and signaling in the nucleus and membrane of plant cells is documented (Stohr et al., 2001; Mur et al., 2013).

There are different types of reactive nitrogen species (RNS) including nitric oxide (NO), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), peroxynitrate (O_2NOO^-), peroxynitric acid (O_2NOOH), nitroxyl anion (NO^-), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and nitrosoglutathione (GSNO). Various types of RNS can cause damage on macromolecules such as carbohydrates, proteins, nucleic acids and lipids (Mur et al., 2013; Szabo et al., 2007).

* Corresponding author.
E-mail address: p-taheri@um.ac.ir (P. Taheri).

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Accumulation of NO might be occurred after infection of plant tissues by different pathovars of *P. syringae* (Delledonne et al., 1998; Zeidler et al., 2004; Zeier et al., 2004; Oliveira et al., 2010), several necrotrophic pathogens (*Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum and Macrophomina phaseolina*; Asai and Yoshioka, 2010; Noorbakhsh and Taheri 2016; Perchepied et al., 2010; Sarkar et al., 2014) and biotrophic fungi (*Golovinomyces orontii* and *Erysiphe pisi*; Schlicht and Kombrink, 2013). Involvement of NO in basal resistance of tomato against *R. solani* is recently reported to be associated with priming lignification, callose deposition and phenolics accumulation (Noorbakhsh and Taheri 2016). Durner et al. (1998) reported that the first enzyme of phenylpropanoid biosynthesis pathway, phenylalanine ammonia lyase (PAL), is induced after infiltration of tobacco leaves and cells with NO donors. NO is known to be involved in cell wall modification as a major defense response of tomato against *Colletotrichum coccodes* (Wang and Higgins 2006). Increased levels of cross-linked proteins and callose production was observed when NO was increased via SNP application (Wang and Higgins, 2006), which is similar to the data obtained in tomato- *R. solani* patghosystem (Noorbakhsh and Taheri, 2016).

R. solani is a necrotrophic fungal pathogen, which causes destructive diseases in different plants especially in bean. It is a main soil borne pathogen in warm and tropical area (Keshavarz-Tohid et al., 2014). This fungal species taxonomically has been divided to 13 anastomosis groups (AG). The genus *Rhizoctonia* has also binucleate species, which are classified in 16 AGs and some of them are used as biocontrol agents against multinucleate *R. solani* (Keshavarz-Tohid and Taheri, 2015). Using biocontrol agents is one of the environmentally safe methods for controlling plant diseases. Various bacteria especially Pseudomonads are excellent candidates for using as biocontrol agents because of their ability to induce resistance in plants and/or antagonistic effects against phytopathogens.

Based on the importance of NO in plant defense, we investigated the role of NO in basal resistance against the necrotrophic fungus *R. solani* and in resistance responses induced by *Pseudomonas fluorescens* strain CHA0 in bean-*R. solani* pathosystem for the first time. Here, for exploring the relation of NO and hydrogen peroxide (H_2O_2), a NO donor (SNAP) and NO scavenger (cPTIO) were used. Also, the role of NO, *P. fluorescens* (CHA0) as biocontrol agent and *R. solani* on H_2O_2 accumulation and activity of antioxidant enzymes was investigated.

2. Materials and methods

2.1. Plant material and inoculation

Kidney bean seeds (cultivar Naaz obtained from Khomein gene bank, Iran) were surface sterilized using 1.2% sodium hypochlorite solution for 4 min and washed twice with sterile distilled water. Then, the seeds were sown in 10 × 6 cm plastic pots containing 50% pitmus and 50% perlit). One seed was sown in each pot and germinated in greenhouse conditions with 20–32 °C temperature and daily light. Four weeks old seedlings were removed from the pots, and then the pitmus and perlit were separated from the roots. Seedling roots were inoculated by putting the roots in bacterial suspension for 20 min. Then, four inoculated seedlings were sown in each pot containing 50% clay and 50% sand. Control plants were inoculated with sterile distilled water. Fungal inoculation was done by putting 0.3 g barley seeds colonized by *R. solani* around the seedling stem base (Nikraftar et al., 2013).

Four weeks old bean seedlings were used for various treatments and the chemicals were sprayed only once on the fully expanded apical leaf of each seedling. The seedlings treated with *P. fluorescens* (CHA0), SNAP and/or cPTIO were inoculated with *R. solani* 24 h (h) after the treatment. All pots were covered with plastic bags for preparing high levels of humidity. Disease severity of inoculated seedlings was investigated based on the disease scales described by Wen et al. (2005). Disease severity on the seedlings was evaluated at 7 days post-inoculation (dpi) using disease scores from 0 to 5 according to the length of necrotic lesions, where 0 = no necrotic lesion on seedling; 1 = necrosis ≤2.5 mm extended; 2 = necrosis 2.5–5.0 mm extended; 3 = lesions ≥5.0 mm extended; 4 = lesions girdling the seedling; and 5 = seedling was damped-off. Finally, the disease index (DI) was calculated as described by Taheri and Tarighi, (2010).

In the detached leaf disc bioassay, which was carried out to determine the role of NO in CHAO-induced resistance in bean-*R. solani* pathosystem, the leaf discs were punched out from the fully expanded apical leaves of 4 weeks old bean plants using a 2-cm diameter cork borer (Taheri and Tarighi, 2011). Each leaf disc was placed on a glass slide in a Petri dish containing a wet filter paper. Pathogen inoculation on the discs and disease evaluation were performed as previously described (Nikraftar et al., 2013). Intensity of disease symptoms was graded into five classes based on the leaf area infected at 5 dpi and the DI was calculated (Taheri and Tarighi, 2010).

2.2. Fungal inocula, bacterial inocula and chemical treatments

R. solani AG 4 HG-II (obtained from Ferdowsi university of Mashhad) was used as the pathogen. The fungal inocula were prepared by placing 5 mm disks (from 5 days refined fungi on potato dextrose agar, PDA) in to 250 mL Erlenmeyer flask containing two times autoclaved barley grains (50 g barley with 30 mL water was autoclaved for 15 min at 121 °C on two consecutive days). The flask was incubated at 26 °C for 12 days. Standard isolate of *Pseudomonas fluorescen* (CHA0), was applied as biocontrol agent. The bacterial isolate was grown on king's B agar medium (KB agar) plates at 26 °C for 24 h. The bacteria were scraped to sterile 25 mM NaCl. Absorption of bacterial suspension was calculated approximately on their OD_{600}. Suspended saline was centrifuged for 5 min at 12,000 rpm. The bacterial pellet resuspended in saline to final density of 5.6×10^8 CFU per mL.

For investigating the role of NO in basal resistance, S-nitroso-*N*-acetylD-penicillamine (SNAP, purchased from Sigma-Aldrich) 200 μM was sprayed on plants as a chemical NO donor. Also, 2-(4-carboxyphenyl)-4,4,5,5-tetramethy-limidazoline-1-oxyl-3-oxide (cPTIO, purchased from Sigma-Aldrich) 200 μM was used for treating bean leaves as NO scavenger (Floryszak-Wieczorek et al., 2006).

To determine the role of NO in CHA0- induced resistance, the leaf discs pretreated with cPTIO were inoculated by CHA0 and *R. solani*. In this assay, first the leaf discs were pretreated with cPTIO (200 μM) for 1 h. In second step, to investigate the ability of CHA0 to induce resistance in the leaf discs pretreated with cPTIO, 5 mm mycelial plug of *R. solani* was inoculated in one side and a spot of CHA0 suspension (OD = 1) was inoculated on another side of each leaf disc to prevent any connection between CHA0 and *R. solani* in the cPTIO + CHA0 + R treatment. The second treatment was pretreated with sterile distilled water and CHA0 and *R. solani* inoculations were performed as mentioned above. The third treatment was pretreated with sterile distilled water and then inoculated with *R. solani*. Control was inoculated with a 5 mm plug of PDA without the fungus. All leaf discs were placed on Lams in Petri dishes containing a wet filter paper. The Petri dishes were kept in an incubator with 24 °C and 12/12 h light/dark photoperiod. Disease severity was evaluated at 48 hpi similar to those previously described.