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Footprint of Nitric oxide in induced systemic resistance

R Acharya, P Patra, N Chakraborty, N S Gupta and K Acharya*
Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany,
University of Calcutta, Kolkata – 700 019, INDIA.

Abstract
Nitric oxide (NO) is a potent signaling molecule with diverse physiological functions in plants. Several rhizobacterial strains may have capacity to induce systemic resistance in (ISR) plants but how far the biochemical mechanisms in which NO participates in this signaling pathway is still an open question. The present study have shown in *Pseudomonas aeruginosa* WS-1 mediated ISR inducing system in *Catharanthus roseus* induces defense enzyme and phenolics and also showed a two fold increase in NO production when challenge with *Alternaria alternata*. Furthermore, NO donor treatment in the host produced same defense molecules in a comparable manner. From those observations it is suggested that NO might have possible signaling role in ISR during crosstalk between the ISR inducing agent and pathogen within the host system.

Key words: *Alternaria alternata*, *Catharanthus roseus*, defense enzymes, Phenolics, *Pseudomonas aeruginosa*.

Other than innate resistance, plants could protect themselves from pathogens through acquired resistance which could be divided into two main categories i.e. systemic acquired resistance (SAR) and induced systemic resistance (ISR). Plants establish SAR when necrotic pathogen tries for an incompatible reaction with the host which is R gene mediated (Van Loon *et al.*, 1998) and ISR is achieved by the host when avirulent pathogen, elicitor molecules, different biocontrol agents, nonpathogenic plant growth promoting rhizobacteria (PGPR) interact with them (Pieterse *et al.*,1996; Van Loon, 2007; Pieterse *et al.*,2007). ISR is important because once induced in plants, may remain stable for a considerable part of their life time (Van Loon *et al.*, 1998). Both ISR and SAR represent a state of enhanced basal resistance of the plant which depends on different signaling molecules (Van Loon, 2007). Elucidation of signaling pathways controlling disease resistance is a major objective in research on plant-pathogen interactions (Pieterse *et al.*, 2007). There are several hypotheses in the signaling pathways of plant defense (Klessig *et al.*, 2000; Nandi *et al.*, 2003; Besson-Bard *et al.*, 2008). Recently, Nitric oxide (NO) has been emerging as a signaling molecule in plant disease resistance (Besson-Bard *et al.*, 2008; Acharya *et al.*, 2005; Acharya and Acharya, 2007; Hong *et al.*, 2008; Acharya *et al.*, 2011a, 2011b). Induction of disease resistance by production of enhanced level of defense enzymes has been reported in several plants to provide protection against invasion of pathogen attack (Friendrich *et al.*,1996; Van Loon *et al.*, 1998). Among the defense molecules, pathogenesis related (PR) protein like peroxidase (PO) plays a key role in biosynthesis of lignin to limit the extent of pathogen spread and also a component of early response in pathogen attack (Bruce and West, 1989), Polyphenol oxidase (PPO) could produce antimicrobial compounds and lignin, while, phenylalanine ammonia lyase (PAL), is a key enzyme in the phenylpropanoid pathway could perform defense related functions (Wen *et al.*, 2005). On the other hand, phenolic compounds are considered to be an important component of the disease defense mechanism (Nicholson and Hammerschmidt, 1992). During early interaction between ISR inducing bacteria and the host, the bacteria must produce one or more signaling compound as they are spatially separated from the inducing agent and giving systemic protection even against foliar pathogens (Kloepper *et al.*, 2004).

The present investigation was undertaken to evaluate the role of NO in the induction of ISR taking *Catharanthus roseus* as a model plant,

Corresponding author:
E-mail:

Pseudomonas aeruginosa WS-1 as ISR inducing agent and *Alternaria alternata* as pathogen, based on the production of different defense molecules as mentioned earlier.

Materials and Methods

Strains

The pathogen, *Alternaria alternata* was isolated from infected *Catharanthus roseus* leaves with typical blight symptoms (Maity *et al.*, 2007). The fungal pathogen was grown on potato dextrose agar (PDA, Himedia, Mumbai, India) medium at 30°C. The biocontrol *P. aeruginosa* WS-1 was obtained from our laboratory culture stalk. The antagonist has subcultured and maintained on triptic soy agar (TSA, Himedia, Mumbai, India) medium for subsequent use.

Treatment

P. aeruginosa WS-1 was used in the induction of defense reaction in *C. roseus* plants. *C. roseus* (two months old) plants were grown in pots (each pot contain one plant) containing sterile soil and maintained at 28±2°C, in the green house. For each pot 100 ml of bacterial suspension at a concentration of 3×10^6 cfu/ml was used to drench the soil. One day after bacterization, one set of bacterized plants were challenged inoculated with spraying of 20 ml of *A. alternata* at a concentration of 3×10^5 cfu/ml / plant (Set- P.a.+A.a.) and another set of bacterized plants was not challenged with pathogen (Set- P.a.). Plants without prior treatment of bacteria were inoculated with pathogen at the same cfu (Set- A.a.). Plants neither treated with bacterial suspension nor challenged by pathogen were kept as control (Set- Control). The humidity of the green house was maintained at around RH 85%.

Enzyme extraction

The fully mature leaf tissues collected from different treated sets after treatment for successive day, were homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant to be used for the enzymatic assay was transferred to a 2 ml vial and stored at -80°C. The standard Bradford assay (1976) was employed, using bovine serum albumin as a standard, to test the protein concentration of each extract.

Peroxidase assay (PO)

Peroxidase activity was assayed spectrophotometrically following the method of Hammerschmidt *et al.* (1982). The reaction mixture consisted of 1.5 ml of pyrogallol, 0.5 ml of enzyme extract 0.5 ml of 1% hydrogen peroxide. The change in absorbance at 420 nm were recorded at each 30 sec intervals for 3 min. the enzyme activity was expressed as changes of absorbance of reaction mixture min^{-1} g^{-1} protein.

Phenylalanine ammonia lyase assay (PAL)

PAL was assayed following the method of Dickerson *et al.* (1984) determining the conversion of L-phenylalanine to transcinnamic acid spectrophotometrically at 290 nm. 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer (pH 8) and 12 mM L-phenylalanine in the in the same buffer for 30 min at 30°C. Enzyme activity was expressed as synthesis of transcinnamic acid (n mol) min^{-1} g^{-1} protein.

Polyphenol oxidase assay (PPO)

PPO activity was determined according to the method of Mayer *et al.* (1965). 200µl of 0.01M catechol was added to the reaction mixture containing 200 µl of enzyme extract and 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5). Enzyme activity was expressed as change in absorbance at 495 nm min^{-1} g^{-1} protein.

Phenol estimation

Leaf samples (1 g) as mentioned earlier were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C (Ziestin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 0.250 ml of 1 N Folin-Ciocalteau reagent and the solution was kept at 25°C. Phenolic content was measured spectrophotometrically at 725 nm using catechol as standard. The amount of phenolics was expressed as µg catechol /g protein.

Nitric oxide estimation

Production of NO was estimated by hemoglobin

assay (Acharya and Acharya, 2007; Hong *et al.*, 2008). 100 mg of leaf tissue was incubated in a reaction mixture containing 10μM L-arginine, 30μM hemoglobin, in a total volume of 2.0 ml of 0.1M phosphate buffer (pH 7.4) at 37°C. Production of NO was measured by using scanning spectrophotometer Hitachi 330 at 575nm as picomolar of NO produced per mg of protein per hour.

Real time NO production was visualized using membrane permeant fluorochrome 4-5-diammino-fluorescein diacetate (DAF-2DA) dye (Bartha *et al.*, 2005). Lower epidermis of leaf was peeled off and placed in a brown bottle containing 1 ml of loading buffer 10 mM KCl, 10 mM Tris HCl (pH 7.2) with DAF-2DA at a final concentration of 10μM for 20 min in dark. Fluorescence was observed with Leica DMLS microscope at excitation wavelength 480nm and emission wavelength 500-600 nm.

Treatment with SNP

Plants were treated with popular NO donor, 100 μM sodium nitroprusside (SNP) by foliar spray and exposed to normal day light. After 24h of treatment mature leaves were harvested, washed thoroughly and utilized for assay of PO, PPO, PAL and phenolics.

Results and discussion

ISR (or) SAR mechanism produces response to local attack by producing defense related compounds thereby reducing or inhibiting further attack by herbivore or pathogens (Hunt *et al.*, 1996; Sticher *et al.*, 1997; Hammer Schimidt, 1999).

Plants have various defense related genes but those are sleeping genes and appropriate stimuli or signals are needed to activate them by prior application of biological inducer which is thought to be a novel plant protection strategy (Radjacommare *et al.*, 2004). In this regard some of these biological control strains like Plant Growth Promoting Rhizobacteria (PGPR) mediated ISR against a broad spectrum of pathogens is being considered as most desirable approach in crop protection (Sticher *et al.*, 1997; Anand *et al.*, 2009). However, the molecular basis of signaling mechanism regarding the development of ISR induced broad spectrum protection is still an open area for research. In the present study defense enzymes systemically induced in host plant by *P. aeruginosa* WS-1 treatment significantly began from 24 h after challenge inoculation, the activity increase further and reached considerably higher on 4th day after treatment. Higher levels of induction of enzymes i.e. PO, PPO, and PAL, were observed in P.a.+A.a. set of *C. roseus* treatment (Fig. 1). Higher accumulation of total phenolics was observed in P.a.+A.a. treated set (Fig. 1). An increased activity of defense enzymes with higher total phenol levels like 26%, 44%, 56% and 23% of PO, PPO, PAL and phenolics respectively over the control were showed in case of P.a.+A.a. treated set after 4th day of incubation. Whereas no marked changes were observed in defense enzymes and total phenolics in untreated (control) as well as *P. aeruginosa* treated plants (P.a. set) which had not been challenged inoculated by the pathogen. In the pathogen treated set only a slight deviation of

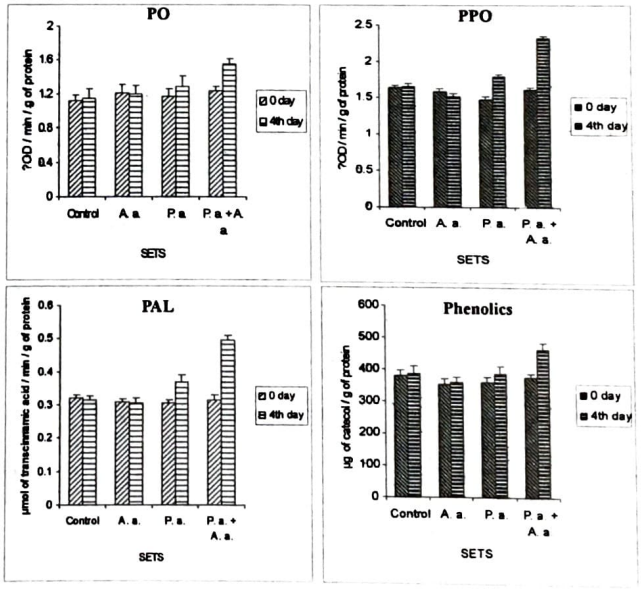

Fig. 1. Effect of application of biocontrol agent *P. aruginosa* WS-1 on the production of PO, PPO, PAL and phenolics on *C. roseus* leaf. The level of enzymes and phenolics production measured on the 4th day of treatment. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*.

defense enzyme activity was observed. According to Sendhil vel (2003) in grapevine plants pretreated with *P. fluorescens*, and Anand *et al.* (2009) in chilli plant pretreated with *Colletotrichum capsici* did not show enhancement of defense molecule production without challenged with respective pathogen. In our case also it is interesting to note that sole application of *P. aeruginosa* WS-1 in the rhizosphere did not insist production of these defense weapons significantly, but enhance the production only when the plant challenged with the pathogen.

A two fold increase in NO production was observed in the ISR inducing system (set- P.a + A.a) on the 4th day after treatment (Fig. 2 Inset). It was further proved by real time NO visualization by using DAF-2DA, a fluorophore widely used for the detection and imaging of NO. DAF- 2DA provide the advantages of sensitivity, specificity and noncytotoxic and permitted the detection of intracellular NO (Lamotte *et al.*, 2004). Fig. 2 shows that ISR system induces NO production

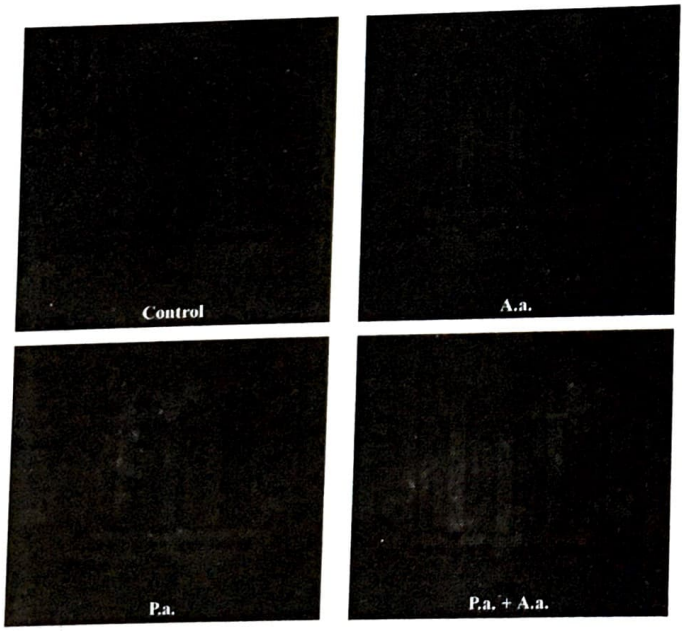

Fig. 2. Nitric oxide visualization in *C. roseus* epidermal cells by DAF 2DA, on the 4th day after treatment of different agents. NO generation is detected by green fluorescence. Inset: Production of nitric oxide by different set. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*. Bar=10 µm.

which was further confirmed by realtime NO generation by DAF- 2DA.

Thus, from the observations it could be speculated that the chemical crosstalk between the ISR inducing agents and the pathogen might inflame the plant to produce elevated level of NO in the system. This observation insisted to investigate whether NO is the candidate taking part in the transduction of the message to produce defense weapons systematically. According to Gauples *et al.* (2008) NO act as a transducer of stress signal in plant system. So, to check the role of NO, we treated plants directly with NO donor SNP and they showed higher production of all those defense molecules (Fig. 3) which suggest that rhizobacteria mediated ISR might be governed by NO but how the signal is perceived and translocate by the plant is still to be investigated.

Our results suggest that activities of defense enzymes and accumulation of phenolics in the host induced by antagonist in response to challenge inoculation with the pathogen and also same system resulted in the induction of NO production. Furthermore, SNP treatment showed the elevation

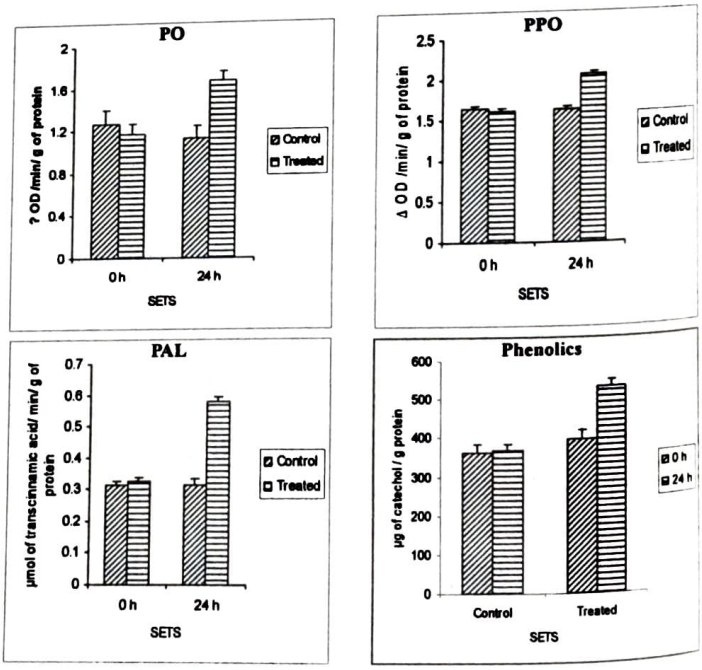

Fig. 3. Effect of SNP (100 µM) on the production of PO, PPO, PAL and phenolics after 24h of treatment. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*.

in the production of same defense molecules over control. In mammals, NO circulate in the blood as either a S-nitroso protein adduct or as low molecular weight S-nitroso thiols such as nitroso glutathione (GSNO). This molecule believed to act as both an intra- and inter-cellular NO carrier, is a powerful inducer of plant defense gene (Durner *et al.*, 1998). Since glutathione is a major metabolite in the phloem (Maria *et al.*, 2003), where the ISR is transmitted, it can be hypothesized that excess NO produced during interaction binds to glutathione; in this form it may act as a long distance ISR signal. All the experimental data suggesting that during ISR, plants may be able to modulate the defense activity and signaling function of this stabilized form of NO.

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