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Involvement of nitric oxide signal in *Alternaria alternata* toxin induced defense response in *Rauvolfia serpentina* Benth. ex Kurz calli

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Abstract

This study elucidates *Alternaria alternata* toxin induced defense enzyme activity and phenolics accumulation and its correlation with nitric oxide (NO) generation. Role of NO in plant defense signaling to biotic stress has been strongly established by earlier investigators. Here, *in vitro* treatment of *Rauvolfia serpentina* Benth. ex Kurz callus with *A. alternata* toxin induced a rapid production of NO at 50 mg L^{-1} toxin concentration. This increase in toxin induced NO production was further confirmed by real time visualization of NO burst using a fluorescent probe, 4, 5-diaminofluorescein diacetate (DAF-2DA) in cells of *R. serpentina*. Parallely, the same concentration of toxin also induced the production of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), β-1, 3 glucanase and phenolics accumulation in the callus tissue of *R. serpentina*. Further, inhibition of NO production by co-treatment with NO synthase inhibitor, N^G -nitro-L-arginine-methyl ester (L-NAME) at 10 µM and NO scavenger, 2-(4- carboxyphenyl)- 4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) at 100 µM reduced the accumulation of all the defense enzymes and total phenols in the callus signifying the regulatory role of NO in the induction of defense enzyme and phenol accumulation. The effect of NO in host defense response was crosschecked further by sole application of NO donor sodium nitroprusside (SNP). Treatment of *R. serpentina* callus with 100 µM SNP showed the similar pattern of defense responses. So, the overall results demonstrated that increased production of NO in *R. serpentina* callus treated with fungal toxin, might act as an essential signaling molecule for triggering the activation of PO, PPO, PAL, β-1, 3 glucanase and phenolics accumulation.

Keywords: Defense enzyme, nitric oxide, sodium nitroprusside, resistance.
Abbreviations: cPTIO_2-(4- carboxyphenyl)- 4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide; DAF-2DA_4,5-diaminofluorescein diacetate; MS_Murashige and Skoog; NO_Nitric oxide; NOS_NO synthase PO_Peroxidase; PPO_Polyphenol oxidase activity; PAL_Phenylalanine ammonia-lyase; L-NAME_N^G -nitro-L-arginine-methyl ester; PR_Pathogenesis-related; SNP_Sodium nitroprusside.

Introduction

Rauvolfia serpentina Benth. ex Kurz., a perennial undershrub (commonly known as 'Sarpagandha' in Sanskrit) is an important member of the family Apocyanaceae. This plant has both medicinal and economic importance and is listed in the Charaka Samhita (c.700 B.C.), an earliest Ayurvedic medicinal text (Kataria and Shekhawat, 2005; Pant and Joshi, 2008). In Ayurvedic medicines, *R. serpentina* was used for treatment of snake-bite, insect stings and in the treatment of mental disorders (Pant and Joshi, 2008). Globally it has drawn exceptional attention in the pharmaceutical industry due to its divergent medicinal properties. However, this plant is severely attacked by leaf blight disease caused by a fungal pathogen *Alternaria alternata* (Maiti et al., 2007) that leads to its significant loss. *A. alternata* is widely distributed in the lower Gangetic plain of West Bengal, India (Maiti et al., 2007). Several species of *Alternaria* are known producers of *Alternaria* mycotoxins- a toxic secondary metabolite (Ostry, 2008). The toxin produced and partially purified from *A. alternata* pathogen of *R. serpentina* was implicated for our present study. Plant tissue and cell culture have been used for the cultivars resistant to pathogen (Koike and Shimada, 1992). Most promising way for the selection of disease resistant lines is to grow callus in the presence of a pathogen culture filtrate or toxins (Behnke, 1979; Thanutong et al., 1983; Carlson, 1973; Svabova and Lebeda, 2005; Kumar et al., 2008). A number of studies have clearly demonstrated a positive correlation between the toxin resistance (at the cellular level) and the resistance to the pathogen (at the plant level) (Ling et al., 1985; Nyange et al., 1995; Jayasankar et al., 2000; Wolpert et al., 2002; El Haldrami et al., 2005; Kumar et al., 2008; Senger et al., 2009). Hence, virulence of the microbial toxin at an under threshold level is responsible for stimulating the host plant defense system. Similar innate immunity induced by microbial toxins has also been reported in animal models (Ottmann et al., 2009). The activation of immunogenic response in the mammalian system by toxins is based on the identification of endogenous compounds that are released in response to the host cell damage and subsequent generation of molecular patterns associated with the damage (Matzinger, 2002; 2007; Ishii et al., 2008). Although, toxins at below threshold level were used for *in vitro* selection of resistance for a longer period, none the less, there is little available knowledge regarding mechanism of the toxin-mediated defense response at the tissue level. Plants exhibit an array of defense mechanisms, by which they defend themselves against different pathogens. They undergo

physiological as well as biochemical changes in response to biotic elicitors produced during host-pathogen interaction (Acharya et al., 2005; Acharya and Acharya, 2007). However, the production of defense enzymes, PR (Pathogenesis-related) proteins and phenols play an important role in the host during pathogen attack and research reports supports the fact that the synthesis and accumulation of PR proteins play an important role in the mechanism of disease resistance in plants (Van Loon, 1997). The coordinated and synchronized response of plants to pathogen or elicitor involves generation of several signals. In recent years, a biologically active molecule, nitric oxide (NO) has been extensively studied as an important signaling molecule in case of plants (Neill et al., 2003; Romero-Puertas et al., 2004). NO has been identified as an important chemical component of plant response to a number of biotic stresses (Durner et al., 1998; Delledonne et al., 1998). This molecule has also been identified as a bio-chemical messenger and has been reported to be associated with different patho-physiological processes (Wendehenne et al., 2004; Shi and Li, 2008). A number of primary literature sources including studies from our own research group have convincingly demonstrated the role of NO in plant defense signaling and in relation to plant disease resistance (Acharya et al., 2005; Acharya and Acharya, 2007; Besson-Bard et al., 2008; Hong et al., 2008; Acharya et al., 2011 a, b) that involves a triggering of resistance-related cellular apoptosis and induction (both local and systemic) of host plant defense genes (Romero-Puertas et al., 2004). Here, an attempt has been made to evaluate toxin - mediated induction of defense response in callus of *R. serpentina* and probable regulatory role of NO in defence responses.

Results

Toxin induced NO release in callus tissue

To monitor the NO generation in toxin treated callus, a spectrophotometric assay was performed using oxyhaemoglobin to methaemoglobin conversion technique (Delledonne et al., 2001). For dose optimization of NO release, calli were treated with various concentration of toxin (0-200 mg L^{-1}) supplemented in liquid Murashige and Skoog (MS) basal media for 1 h. After the incubation period, washed calli were used for the measurement of NOS activity. As demonstrated in Fig. 1A, the effective toxin concentration was investigated when a sharp increase in the NO release was observed at 50 mg L^{-1} toxin concentration showing a 2.42 fold increase over the corresponding control following which the NO production gradually declined. Furthermore, the kinetics of the NO production over time was determined at the optimized toxin concentration of 50 mg L^{-1}. A strong surge in the amount of methaemoglobin indicated a rapid NO accumulation when the plant calli was exposed to toxin. This sudden surge; however, hit the plateau stage at 3 h post toxin treatment and demonstrated a peak at 6 h with 10.0 µmol $h^{-1}g$ $protein^{-1}$ of NO production (Fig.1B). However, there was a gradual decline in the NO release post 6 h toxin treatment of calli.

Visualization of NO burst using DAF-2D stain

The real time NO production was visualized after application of a specific, non-cytotoxic and membrane-permeable fluorescent indicator dye (DAF-2DA). The reaction between NO and DAF- 2DA generates a fluorescent green colored triazole derivative called DAF-2T (Kojama et al., 1998). This fluorescent indicator allows convenient and accurate measurement of NO production in the eukaryotic cells (Foissner et al., 2000; Gould et al., 2003). The calli cell suspensions were loaded with DAF-2DA dye and then observed under confocal laser scanning microscope. The basal fluorescence presented in the Fig. 2A was generated as a result of long time incubation of control cells indicating basal NO synthesis. In contrast, the incorporation of toxin during image acquisition initiated rapid glow of fluorescence suggesting high NO synthesis (Fig. 2B) and continued for longer time period. The toxin induced NO burst was completely inhibited by L-NAME (methyl ester derivative of the NOS substrate L- arginine) (Fig. 2C) suggesting the increased production of NO has been as result of the activation of NOS- like enzyme. The NO scavengers PTIO and its corresponding derivative cPTIO are commonly used to identify NO involvement in plant processes (Planchet et al., 2006). Indeed, co-infiltration of the NO scavenger, cPTIO with toxin did not show any detectable NO emission even after prolonged period (Fig. 2D).

Effect of toxin, cPTIO, L-NAME, SNP on defense enzyme and polyphenol accumulation

The optimized toxin concentration i.e. 50 mg L^{-1} was used in subsequent experiment. Toxin triggered approximately a 1.6 fold increase in PO activity in callus tissue, which peaked at 9 h (Fig. 3A). The toxin induced increase in PO activity was suppressed in combination with L-NAME (NO synthase inhibitor) or cPTIO (NO scavenger). The NO donor sodium nitroprusside (SNP) itself stimulated the PO activity 1.7 fold at 9 h after treatment (Fig. 3B). There was a marked increase in the PPO activity in the toxin treated callus. The activity level reached to maximum at 9-12 h after treatment and the increased level was approximately 1.7 fold and was maintained over the control throughout the experimental period (Fig. 4A). The combination with NO inhibitor/NO scavenger separately in the incubation mixture suppressed the PPO activity. Furthermore, the sole application of NO donor stimulated the PPO activity 1.8 fold compared to that of control (Fig. 4B). A gradual increase in intracellular PAL activity in *R. serpentina* calli was observed upon treatment with toxin, reaching its peak at post 9 h treatment. The peak was found to be 1.9 fold over the corresponding control (Fig. 5A). However, the toxin induced PAL activity was suppressed both by toxin supplemented with L-NAME or cPTIO. This effect was again reversed by NO donor stimulating the PAL activity ~ 2 fold over that of control (Fig. 5B). The activity of ß-1, 3 glucanase at different time intervals are presented in Fig. 6A. In case of the control, the ß-1, 3 glucanase activity did not show any apparent change over 24 h. The highest enzyme activity was reached at 12 h post treatment with toxin showing 1.9 fold increase over control. Then a subsequent decrease in the enzyme activity was observed and the higher activity level was maintained up to the end of the experiment. The NO donor itself activates the ß-1, 3 glucanase activity showing ~ 2 fold increase at 12 h post treatment. Treatment of calli with toxin in combination with L-NAME/cPTIO reduced the enzyme activity (Fig. 6B). The total phenol content also increased in the toxin treated calli, reaching maximum at 9 h post treatment and this increased level was maintained throughout the experimental period (Fig. 7A). Like earlier results both the NOS inhibitor and NO scavenger reduced total phenol content in the calli. The NO donor increased the level of total phenol like the toxin treated sets 2.1 fold over that of the control (Fig. 7B).

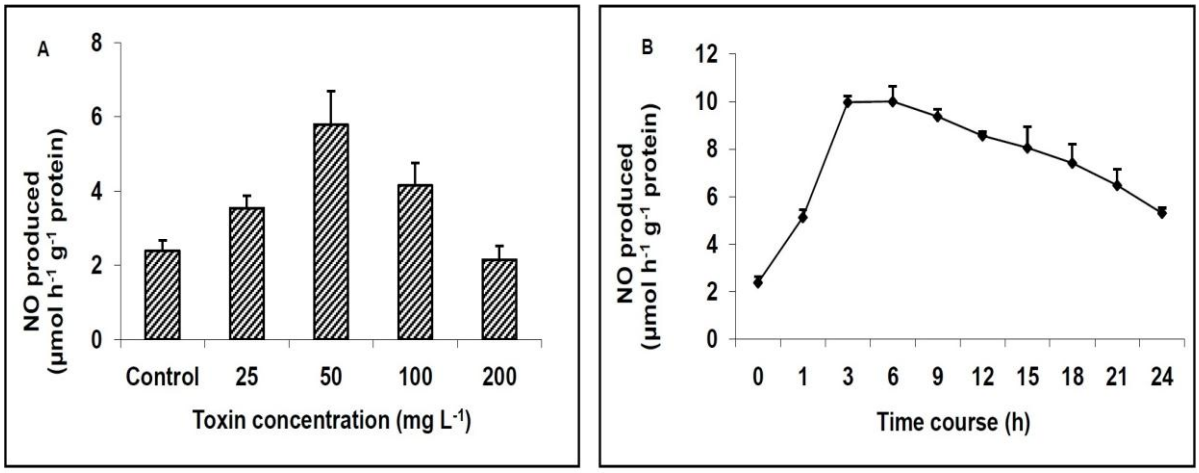

Fig 1. NO production in callus of *R. serpentina*. (A) Treatment with toxin at various doses (B) Time course analysis. Results are mean ± SD of three separate experiments, each in triplicate.

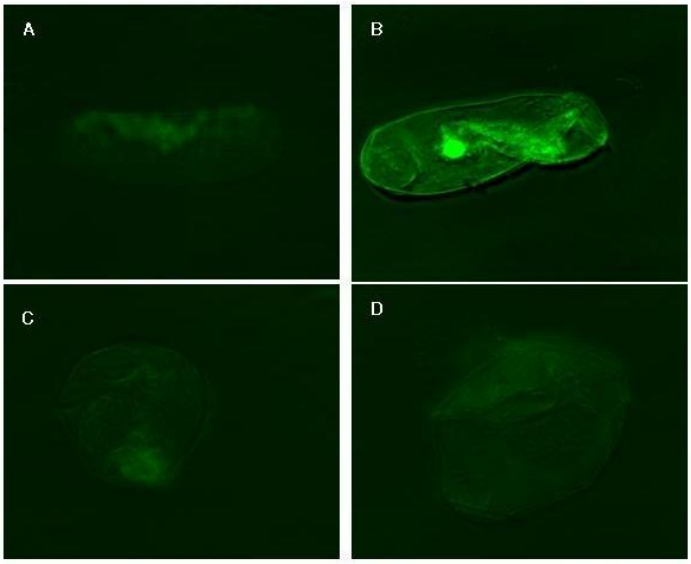

Fig 2. Effect of toxin, inhibitor of NOS and NO scavenger on fluorescence signal in living *R. serpentina* cells loaded with 10 µM DAF-2DA for 10 min. The images were recorded through image processor. (A) A weak signal was detected in the cell incubated with DAF-2DA (B) The fluorescence intensity was strengthened by treatment of cell with 50 mg L^{-1} *A. alternata* toxin (C) but obviously weakened by addition of 10 µM L-NAME, (D) and 100 µM cPTIO.

Discussion

We previously reported that the level of NO in plant is the key determinant of resistance and susceptibility (Acharya and Acharya, 2007). Elevation in NO level was studied in *Raphanus sativus* by elicitors (Acharya et al., 2011b) and in tobacco by cryptogein (Lamotte et al., 2004) showing its involvement in the signal transduction process leading to induced defense responses. In the present study we investigated the optimum concentration of *A. alternata* toxin mediated increased NO production in *R. serpentina* callus and analyzed NO function in the elicitor signaling pathway. Results showed that the optimum concentration of *A. alternata* toxin for elevated NO production was 50 mg L^{-1}, beyond which NO production declined. Similarly, Yang et al. (2012) observed that *A. alternata* toxin had phytotoxicity on rose leaves at >50 mg L^{-1} concentration. NO production was further monitored using DAF-2DA, a fluorophore widely used for detection and imaging of NO. DAF-2DA had the advantages of sensitivity, specificity and non-cytotoxicity and permitted the detection of intracellular NO. In order to assess the extent of induction of defense enzymes and phenols at the optimized concentration of toxin in *R. serpentina* callus, PO, PPO, PAL, β-1, 3 glucanase and phenols level were measured at various incubation time interval. All the enzyme activity and phenol level in the callus tissue reached its peak at 9-12 h after challenged with *A. alternata* toxin. Previously, Montesano et al. (2003) reported that fungal toxins can induce resistance related metabolites in plants, including different oligosaccharides, lipids, peptides, proteins etc. and is believed to enable plants to resist phytopathogen attack. Our results also coincides with the recent findings where an increase in PO, PAL and phenol level was observed in calli (*Sesamum prostratum* against *Fusarium oxysporum f. sesame*) treated with crude toxin (Rajab et al., 2009). Furthermore, Zhang et al. (2003) showed the ability of *Verticillium dahliae* toxin that increased β-1, 3 glucanase activity induced defense response in cotton

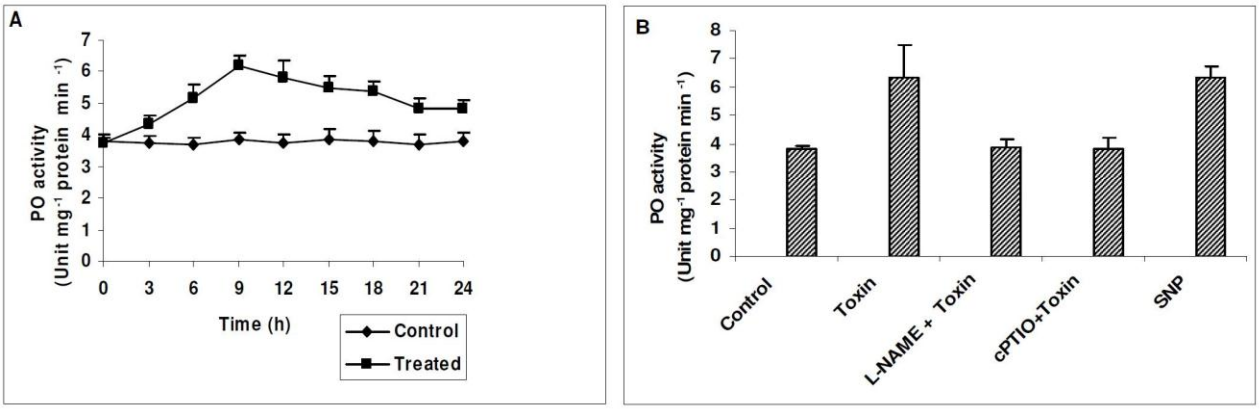

Fig 3. PO activity in callus of *R. serpentina.* (A)Time course analysis in callus treated with toxin. (B) Effect of Toxin, Toxin with L-NAME, Toxin with cPTIO and SNP on PO activity. Results are mean ± SD of three separate experiments, each in triplicate.

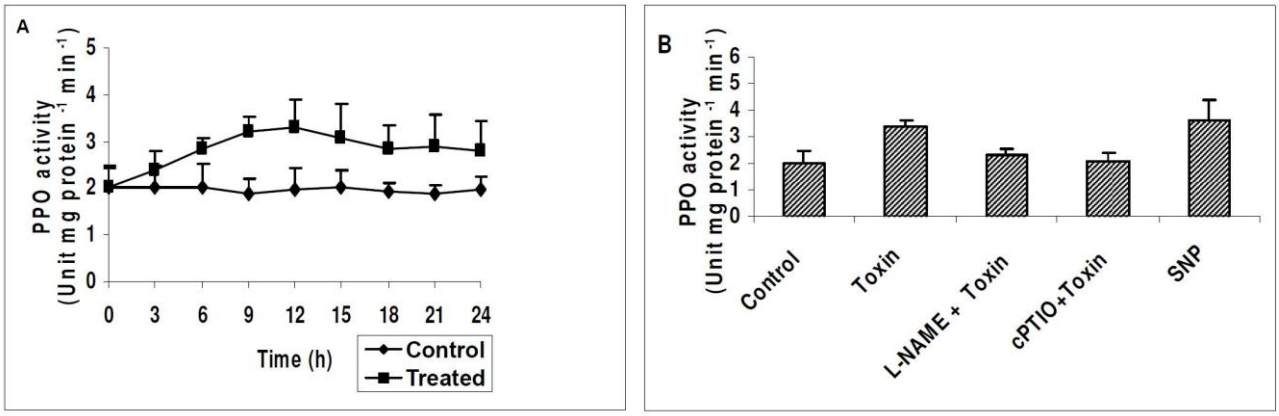

Fig 4. PPO activity in callus of *R. serpentina.* (A) Time course analysis in callus treated with toxin. (B) Effect of Toxin, Toxin with L-NAME, Toxin with cPTIO and SNP on PPO activity. Results are mean ± SD of three separate experiments, each in triplicate.

cultivars. To correlate the relationship between increased production of NO and elevated level of defense enzymes and phenols in the toxin treated callus tissue, co-treatment of *A. alternata* toxin with NOS inhibitor (L-NAME) or NO scavenger (cPTIO) reduced the increased NO production to basal level in *R. serpentina* callus showing a strong unidirectional correlation with plant defense regulators. Similar type of observation was recorded by Zhao et al. (2007) where co-treatment of cells with cPTIO and oligochitosan did not increase PAL activity in tobacco. The overall results and observations suggest that NO production might be a crucial step for toxin mediated improvement of defense responses. The role of NO in plant defense signaling has been extensively unveiled and represented as an important element for plant responding to biotic stresses (Durner et al., 1998, Delledonne et al., 1998, Acharya et al., 2005, Acharya et al., 2011b). In the present work direct addition of the NO donor SNP induced the production of PO, PPO, PAL, β-1, 3 glucanase and phenols in the *R. serpentina* callus. Durner et al. (1998) reported that injection of tobacco seedling with mammalian NOS induced the transcription of PR genes and application of NO donor GSNO in tobacco cell culture also induced the transcription of these genes. Application of NO via NO donor SNP in rice cell suspension culture induced the expression of *pal*, *prl* and *cha* genes (Hu et al., 2003) and PAL in *Taxus chinensis* suspension cells (Maojun et al., 2004) and tobacco (Zhao et al., 2007). So, all the accumulated data from the present investigation indicates that *A. alternata* toxin induced NO production is an essential signal molecule for triggering the toxin induced PO, PPO, PAL, β-1, 3 glucanase activity and phenols production in *R. serpentina* callus. The relationship between NO and other signal molecules needs to be further investigated.

Materials and Methods

Chemicals

4, 5-diaminofluorescein diacetate, N^G -nitro-L-arginine-methyl ester; Sodium nitroprusside, 2-(4- carboxyphenyl) -4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide were chemicals and solvents are of analytical grade.

Plant materials

One month old healthy *Rauvolfia serpentina* plants were collected from Medicinal Plant Garden, Ramakrishna Mission Ashrama, Narendrapur, Kolkata, India.

Callus culture

Nodal explant from *in vitro* generated shoot of *R. serpentina* were cultured in MS (Murashige and Skoog, 1962) basal media supplemented with various concentration of 2, 4-D (0.125-2.0 mg L^{-1}) alone or 2, 4-D in combination with Kinetin (0.1-1.6 mg L^{-1}). The pH of the culture media was

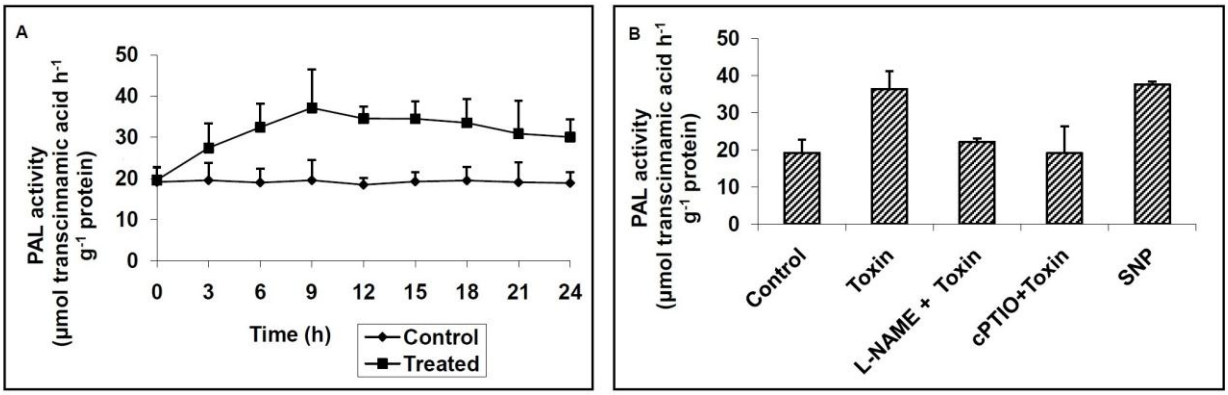

Fig 5. PAL activity in callus of *R. serpentina.* (A) Time course analysis in callus treated with toxin. (B) Effect of Toxin, Toxin with L-NAME, Toxin with cPTIO and SNP on PAL activity. Results are mean ± SD of three separate experiments, each in triplicate.

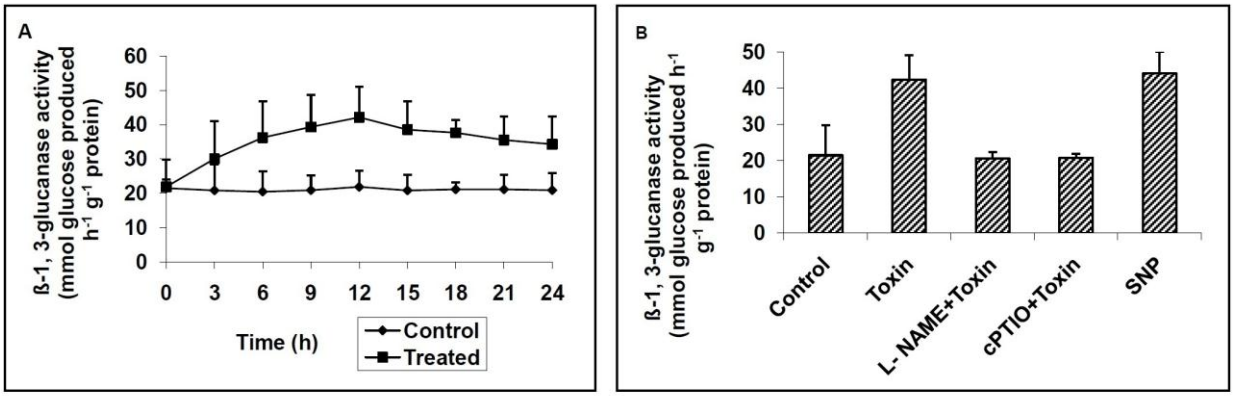

Fig 6. ß-1, 3glucanase activity in callus of *R. serpentina.* (A)Time course analysis in callus treated with toxin. (B) Effect of Toxin, Toxin with L-NAME, Toxin with cPTIO and SNP on ß-1, 3glucanase activity. Results are mean ± SD of three separate experiments, each in triplicate.

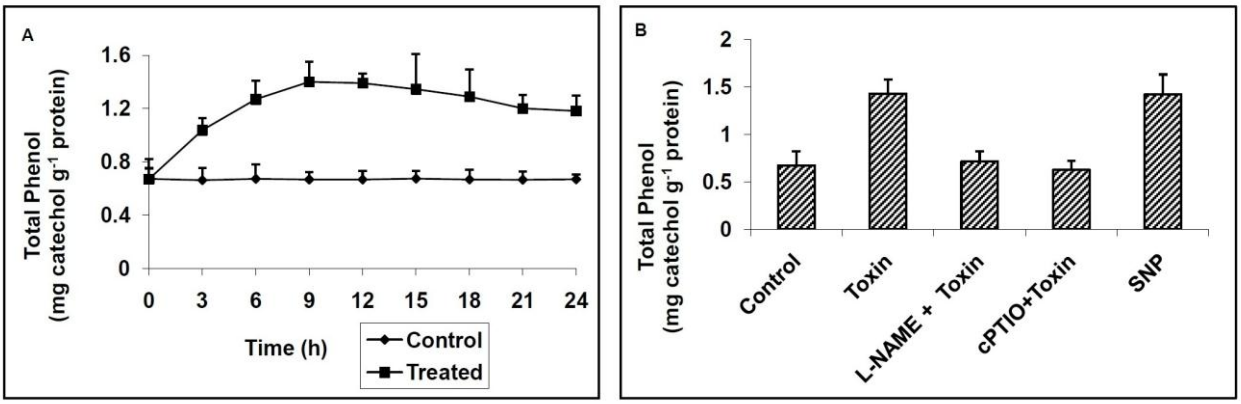

Fig 7. Total phenol content in callus of *R. serpentina*. (A)Time course analysis in callus treated with toxin. (B) Effect of Toxin, Toxin with L-NAME, Toxin with cPTIO and SNP on total phenol content. Results are mean ± SD of three separate experiments, each in triplicate.

adjusted to 5.8 before adding agar (0.8%) and was autoclaved for 20 min at 121°C. Cultures were incubated under 16 h photoperiod (using cool white fluorescent light) 60% RH and 25±1°C. Calli induced were sub cultured at 4 week interval.

Alternata alternata toxin production and purification

A. alternata was obtained in pure mycelial culture from the diseased leaf tissue of *R. serpentina*. The pure culture of the pathogen was maintained on PDA (Potato Dextrose Agar) slants. The pathogen was cultured in Richard's Medium (Thanutong et al., 1983; Slavov et al., 2004). Toxin was partially purified according to the protocol mentioned by Slavov et al. (2004) with some modification.

Assay of NO formation

NO was estimated by haemoglobin assay method (Delledonne et al., 2001). Initially the calli pieces were incubated aseptically with liquid MS basal media supplemented with varying concentration of toxin (0-200 mg L^{-1}) under controlled light and temperature (25±2°C) for 1 h. After incubation the calli were washed thoroughly with

sterilized distilled water and incubated in the reaction mixture containing 10 µM L-arginine and 10 µM haemoglobin in a total volume of 2 mL of 0.2 M phosphate buffer (pH 7.4). The OD was measured in a UV-Vis spectrophotometer (HITACHI-1130 spectrophotometer) at 401 nm and NO was calculated using an extinction coefficient of 38,600 M^{-1} cm^{-1} (Salter and Knowles, 1998). For Fluorimetric assay of NO generation, suspension cells prepared from *R. serpentina* calli was exploited. Suspension cells were treated with (a) Toxin (50 mg L^{-1}), (b) Toxin (50 mg L^{-1}) + L-NAME (10 µM), (c) Toxin (50 mg L^{-1}) + cPTIO (100 µM) supplemented in liquid MS medium and calli in only liquid MS medium served as control. Post 6 h incubation, the samples were washed with sterile fresh medium and were incubated in DAF-2DA dye (10 µM) for 15 min in the dark. Confocal fluorescent images were captured on the BD pathway at 855 bioimaging system (BD Biosciences) under excitation wavelength 488 nm and emission wavelength 530 nm Long-pass.

Treatment of R. serpentina callus for enzyme assay and total phenol estimation

Calli pieces were transferred into 100 mL conical flask containing liquid MS medium supplemented with (a) Toxin 50 mg L^{-1}, (b) Toxin 50 mg L^{-1} + cPTIO (100 µM), (c) Toxin (50 mg L^{-1}) + L-NAME (10 µM), (d) SNP (100 µM). Calli in only liquid MS medium served as control. The flasks were incubated under controlled light and temperature (25±2°C) on a rotary shaker.

Extraction of enzyme

Callus were harvested after a period of incubation and homogenized with liquid nitrogen. 250 mg of the powdered sample was extracted with 2 mL of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was then centrifuged for 20 min at 12000 rpm at 4°C. The supernatant was transferred in 2 mL vials and stored at -80°C.

Estimation of protein

The concentration of protein in the enzyme extract was determined following Bradford method (1976) using Bovine serum albumin as standard.

Spectrophotometric assay of enzymes

Peroxidase (PO) was estimated as per the method of Hammerschmidt et al. (1982). Reaction mixture consisted of 0.5 mL of crude enzyme extract, 1.5 mL of pyragallol and 0.5 mL of hydrogen peroxide (1%). The change in absorbance at 420 nm was recorded at 30s intervals for 3 min. PO activity was expressed in Umg^{-1}protein (U= change in 0.1 absorbance min^{-1} mg^{-1} protein).

Polyphenoloxidase (PPO) activity was assayed following the protocol of Mayer et al. (1965). The assay mixture consisted of 0.2 mL of enzyme extract, 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 mL of 0.01 M catechol. PPO activity was determined by the increase in absorbance at 495nm. Activity was expressed in Umg^{-1}protein (U= change in 0.1 absorbance min^{-1} mg^{-1} protein)

Phenylalanine ammonia-lyase (PAL) was determined as per the method of Dickerson et al. (1984). The assay mixture consisted of 0.4 mL of enzyme extract, 12 mM L-phenylalanine and 0.5 mL of 0.1 M borate buffer (pH 8). The mixture was incubated for 30 min at 30°C. The enzyme activity was expressed as synthesis of transcinnamic acid (µ mol h^{-1}g $protein^{-1}$).

ß-1,3 glucanase activity was spectrophotometrically determined following the method of Pan et al. (1991).The assay mixture consisted of 62.5 µL enzyme extract 62.5 µL of (4%) laminarin followed by incubation of the mixture for 30 min at room temperature. The reaction was stopped by adding 375 µL of dinitrosalicylic acid followed by heating for 10 min on boiling water bath. Then to the resulting solution distilled water was added. The absorbance was recorded at 520 nm and the enzyme activity was expressed as mmol glucose produced h^{-1}g $protein^{-1}$.

Quantification of total phenol

The total phenolic content was estimated following the method of Zieslin and Ben-zaken (1993). The treated calli tissue (1g) were homogenized in 10 mL of 80% methanol for 15 min at 70°C. The resulting methanolic extract was used for estimation of phenol. The reaction mixture consisted of 1 mL of methanolic extract, 5 mL of distilled water and 250 µL of 1N Folin-Ciocalteau reagent followed by its incubation at 25°C. Total phenols was expressed as mg catechol g $protein^{-1}$.

Statistical analysis

Statistical analyses was performed by student's 't' test and in all the cases results are mean ± SD (standard deviation) of at least three individual experimental data.

Conclusion

The present study demonstrated the toxin induced generation of NO and activity of PO, PPO, PAL, ß-1, 3 glucanase and phenol accumulation in *R. serpentina* callus. These finding together with the effect of NO donor (SNP), co-treatment of NO synthase inhibitors L-NAME /NO scavenger (cPTIO) with toxin on defense enzyme activity and phenol accumulation justifies the possible signaling role of NO in toxin induced defense response in *R. serpentina* calli.

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