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Alternaria alternata toxin mediated elicitation of secondary metabolite pathway in *Rauvolfia serpentina* along with disease protection

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ABSTRACT

The present study aimed to investigate the elicitation of secondary metabolites in Rauvolfia serpentina by Alternaria alternata crude toxin that serves as an elicitor. R. serpentina plants were treated with toxin and subsequently challenge inoculated with the conidial suspension (10^5 conidia mL^{-1}) of A. alternata. Foliar application of toxin was repeated at fifteen days interval. The increased production of PAL and NO after toxin treatment suggested the capacity of toxin to induce the production of secondary metabolites which was further justified with the increased production of reserpine. Besides this toxin was quite effective in reducing the disease severity, increasing root biomass and maintaining good health of the plants. Henceforth the present analysis suggests the efficacy of toxin in mediating the elicitation of medicinally important secondary metabolite reserpine in one hand along with its positive impact in disease protection and health of R. serpentina.

Keywords bio control, disease incidence, reserpine, root, toxin

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INTRODUCTION

Plants are the major source of pharmaceuticals. Chemically most of these pharmaceuticals are secondary metabolites. *Rauvolfia serpentina* a commercially important medicinal plant is an important source of alkaloid reserpine. Reserpine is an indole alkaloids, recognized as one of the most active compound exhibiting several therapeutic activities including antipsychotic, cardiovascular disorder, antihypertensive, rheumatism and snake bite [1, 2]. Because of its immense therapeutic importance it is in great demand at the global level.

It has been widely recognized that the secondary metabolite production and plant defense response had a strong correlation. Plants utilize a wide array of defense mechanism against all sorts of biotic and abiotic stress including activation and biosynthesis of secondary metabolites [3]. Elicitation of plant cells represents an important biotechnological method to improve the production of these valuable metabolites. Use of fungal elicitors is one of the effective strategies for induction of useful secondary metabolites in medicinal plants. For instance an increase in level of indole alkaloids upon elicitation with fungal cell wall fragments was observed in cell suspension of *Catharanthus roseus* [4, 5]. Similarly, fungal mycelia induced an increased production of diosgenin in cells of *Dioscorea deltoidea* [6]. Cells of *Taxus chinensis* treated with endophytic fungus induced a raise in accumulation of taxol [7].

Toxin has now been recognized as elicitor because of their capacity to elicit plant defense arsenals by inducing several resistance related responses. For example a toxin-Picolinic acid secreted by *Magnaporthe grisea* triggers

oxidative burst and hypersensitive reaction (HR) in rice leaf, resulting in induction of resistance to succeeding inoculation with pathogen virulent spore [8]. Similar type of observation was also shown by Stone *et al*. [9] where fumonisin B1 acts as an elicitor, inducing active defense in maize. Several previous reports also support the elicitor like activity of toxin in various plants [8, 10-14].

Toxin modulates the activation of various defense molecules including phenylalanine ammonia lyase (PAL). PAL is an important key enzyme of phenylpropanoid pathway vital for biosynthesis of secondary metabolites [15, 16]. In phenylpropanoid pathway, PAL catalyzes the synthesis of transcinnamic acid through non-oxidative deamination of L-phenylalanine, producing precursors for a wide range of secondary metabolites [16] in plants.

In recent times, it has been established by many workers that there is a direct correlation between the activation of defense arsenal in plants with the production of nitric oxide, an important signaling molecule in biological system [17-23]. Our previous work also coincides with the earlier literatures where we have shown that *Alternaria alternata* crude toxin mediated elicitation of defense enzymes have also a strong relation with NO production [13, 14]. It has also been established by many workers that the NO production is linked with the triggering in the biosynthesis of pharmaceutically important secondary metabolites in plants as for example, ginseng saponin [24], puerarin [25], hypericin [26], catharanthine [27], taxanes [28] and artemisinin [29].

Here, for the first time, an attempt has been made to understand the efficacy of toxin in elicitation of secondary metabolites in *Rauvolfia serpentina* along with the protection of plant from the blight disease after challenge inoculation with the pathogen *Alternaria alternata.*

MATERIALS AND METHODS

Plant material

In vitro regenerated plantlets of *R. serpentina*, hardened, acclimatized in green house following the method of Gupta and Acharya [14]. Two months old healthy potted plants were utilized for further study.

Pathogen and toxin preparation

For crude toxin preparation the pathogen, *A. alternata* was cultured in liquid Richard's medium [30, 31] and incubated for three weeks at 25°C in darkness. Mycelial mats were separated from the filtrate. Toxin was isolated as following the method of Slavov *et al*. [30].

Preparation of *Alternaria alternata* conidial suspension

To prepare conidial suspension, fresh isolates of *A. alternata* grown on PDA media was scrapped using sterile scalpels and were transferred into sterile distilled water. The suspension of conidia was adjusted to approximately 10^5 conidia mL^{-1} using a haemocytometer.

In planta treatment

Two month old potted plants were treated with toxin at a concentration of 50 mg L^{-1} by foliar application and were challenge inoculated with freshly grown conidial suspension (10^5 conidia mL^{-1}) of *A. alternata*, once, 48 h after the first treatment. Then plants were covered with sterile polythene sheets to allow the pathogen to establish. Toxin treatment (50 mg L^{-1}) was repeated at 15 days interval until harvesting. Month wise disease index was recorded following the 0-5 disease rating scale [32] for a period of March to August. Lesions for 0-5 rating for individual leaf were presented in Figure 1.

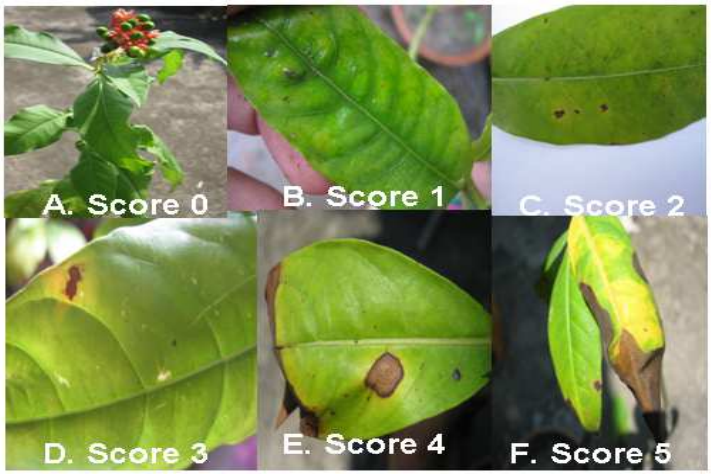

Figure 1: A-F. Showing disease score on a 0-5 scale
0=no infection; 1=single small brown spot only on upper surface; 2=2-3 spots with yellow boarder on upper surface, impression of brownish colour appearance on the lower surface; 3=spots appeared on both surfaces, size larger than the grade 2; 4=spots coalesced to form larger lesions with distinct concentric rings; 5=coalesced lesions form larger irregular lesions, whole leaf turned yellow.

Estimation of PAL
Phenylalanine ammonia lyase (PAL) was assayed per the method of Dickerson *et al*. [33]. The reaction mixture includes 0.4 mL of the enzyme extract, 12 mM L-phenylalanine and 0.5 mL of 0.1 M borate buffer (pH 8). The reaction mixture was incubated for 30 min at 30°C. The PAL activity of the treated plants were compared with the untreated control.

NO Assay
Nitric oxide was estimated using haemoglobin assay method of Delledonne *et al.* [34]. Leaf samples from treated and control plants were harvested 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 [35]. Increase in the production of NO was compared with appropriate control sets.

Extraction and estimation of reserpine content in roots of *R. serpentina*
After six months roots of both toxin treated and control plants of *R. serpentina* were harvested, properly washed under running tap water, dried (sun dry) and weighed. 100 mg of the dried root sample was extracted with methanol and HCl (98:2). Reserpine was quantified by HPLC (Perkin-Elmer, USA) analysis using 250 mm x 4.6 mm Thermo C18 column; 268 nm detector. Reserpine was separated using a flow rate 1ml/min with acetonitrile and phosphate buffer (35:65 v/v) as mobile phase [36]. The retention time was 17.4 min for reserpine. The reserpine content was estimated in samples using reserpine standard.

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**.**

RESULTS AND DISCUSSION

In our previous report we had demonstrated *A. alternata* toxin induced defense responses in *R. serpentina* both in callus and in planta, where all the defense molecules peaked at 48 hrs after treatment [13, 14]. In the present study the toxin treated plants were challenge inoculated with conidial suspension (10^5 conidia mL^{-1}) of *A. alternata* after 48 hr of first toxin treatment. Every fifteen days interval toxin treatment was repeated. Experiment on foliar application of toxin was conducted during the month of March to August. Three consecutive trials were made showing similar trends throughout the experimental period. After each treatment with toxin the level of PAL and nitric oxide (NO) was measured every 48 hr. It was observed that in treated plants the PAL activity was significantly higher each time which ranges from 3.7 to 4.3 times over the control. An increase in NO production was also

observed by 3.9 ± 0.4 fold in comparison to control. Simultaneously, we had measured disease score every thirty days interval starting from the first treatment. From the data it is evident that the index value in the untreated plants reached maximum to 4.8, where more or less all plants were severely affected. On the contrary in treated set, the maximum value was nearly two, showing around sixty percent reduction in disease severity (Table 1, Figure 1).

These results might be correlated with earlier studies where treatment with toxin induced an increased production of defense molecules [13, 14]. Similar type of observation was recorded by other workers as well. For example treatment of *Avena sativa* with lower concentration of host specific toxin, victorin produced by the fungal pathogen *Helminthosporium victoriae* elicits accumulation of a phytoalexin avenalumin in oat plant [37]. Later, Navarre and Wolpert [38] suggested that the toxin victorin at low concentration may serve as an elicitor by Ca^{2+} and ethylene signalling. Another toxin, picolinic acid, produced by rice blast fungal pathogen has shown to elicit hypersensitive responses (HR) and improve disease resistance in rice crop [8]. Stone *et al.* [9] reported that fumonisin B1 served as an elicitor and have capacity in induction of several active defense responses in maize crop. Among these defense molecules PAL plays a critical role in defense reaction. An increase in PAL activity was observed by Rajab *et al*. [39] in calli of *Sesamum prostratum* against *Fusarium oxysporum f. sesame*) treated with crude toxin. The increase in PAL activity can be correlated with secondary metabolite production [40]. As PAL catalyzes the conversion of phenylalanine into cinnamic acid, the earliest committed step in the phenylpropanoid pathway [41], that produce a number of phenolic compounds with defense-related role. This in turn is very much linked with the production of secondary metabolites, as the derivatives of cinnamic acids are the precursors for a broad variety of secondary metabolites. During these processes, various signal molecules including nitric oxide [13, 14, 42] play a crucial role in inducing intracellular defense responses in plants. For instance a stronger NO burst was reported in response to VD-Toxin in cotton and *Arabidopsis*, showing that NO play an important role in the induction of resistance [43, 44] in plants.

Table 1: Efficacy of *Alternaria alternata* toxin for the control of leaf blight disease of *Rauvolfia serpentina.* Disease index was rated on 0-5 scale

Number of months	Number of treatments	Disease index	
		Control	Treated
1	1	1.06±0.07	1.03±0.06
2	2	1.17±0.06	1.10±0.10
3	3	2.09±0.05	1.98±0.08
4	4	2.60±0.10	2.02±0.08
5	5	4.03±0.05	2.03±0.11
6	6	4.80±0.05	2.03±0.05

Furthermore, the health of the treated plants showed a prominent difference from the control (Figure 2). In treated plants the healthy appearance might be correlated with the lower disease incidence and also increased production of NO. Hyat *et al.* [45] reported that NO as a non traditional phytohormone that might function as a gaseous endogenous growth regulator. Xu *et al*. [46] showed treatment of *Catharanthus roseus* with SNP (NO donor) increased the biomass of cells. After six months of treatment, roots were harvested from the potted plants. An increase in root volume and biomass was observed in toxin treated plants in 2 fold (Figure 3). Similar observation was made by Tiwari *et al*. [47] in *Panax ginseng* plant. Their observation suggested that NO might act downstream of auxin action in the process of root growth and development.

Figure 2: Morphology of plants of *Rauvolfia serpentina* under pot experiment. (A) control; (B) toxin treated

Figure 3: Morphology of roots of *Rauvolfia serpentina* under pot experiment. (A) control; (B) toxin treated

The increase in secondary metabolites is an important aspect of defense response against pathogenic attack in plants [1], which can also be induced by elicitors or signal molecules [48]. *R. serpentina* is reported to be a rich source of alkaloids that are mostly located in roots. The quantitative estimation of reserpine content from roots of *R. serpentina* was performed by HPLC technique. The total time of analysis was 30 min. Detection of the alkaloid in control and treated root samples were estimated by comparing the retention time (17.4 min) and λmax (216, 267 and 296 nm) of reserpine standard (Figure 4 A). From Figure 4B and 4C it was evident that reserpine was detected in both root samples at the same retention time. Nearly 1.3 fold increase in reserpine content was noted compared to control (Figure 5). The result elucidates the efficacy of toxin in eliciting secondary metabolite accumulation upon treatment with toxin in roots of *R. serpentina*. The elicitor induced increase in reserpine content had been reported in somatic embryos of *R. serpentina* elicited with methyl jasmonate by Harisaranraj *et al*. [1].

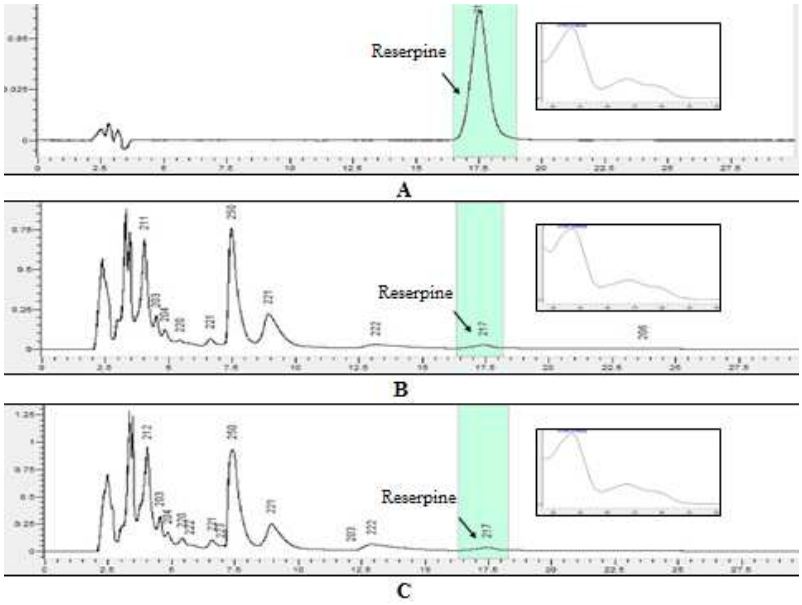

Figure 4: HPLC chromatogram showing reserpine profile of methanolic extract of roots of *Rauvolfia serpentina.* (A) standard reserpine; (B) control set; (C) toxin treated set

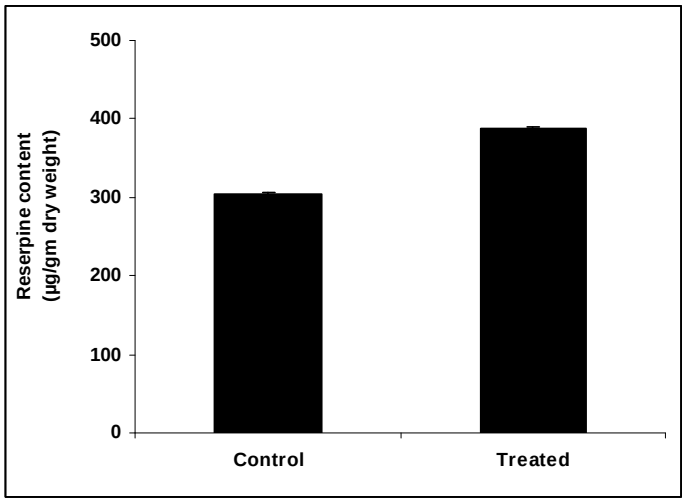

Figure 5: Effect of toxin on accumulation of reserpine in roots of *Rauvolfia serpentina* under pot experiment

CONCLUSION

In conclusion, the present work was undertaken to verify the bio efficacy of the toxic metabolite to combat blight disease in *R. serpentina* plants and its effect on plant health, root biomass and reserpine content. The present result elucidated the effect of toxin on reducing disease severity in *R. serpentina* against blight disease caused by *A. alternata*. The toxin here served as an elicitor, provoking the defense system in the plant thereby declining or delaying the symptoms of the blight disease in the foliar portion of the plantlets. Hence, exploration and effective use of this toxic metabolite as an agent would serve as ideal tools for controlling blight disease in *R. serpentina.* This method would facilitate to reduce the disease incidence by decreasing the susceptibility of the plant against the pathogen. So, far bets of our knowledge, this might be the first report where application of toxin in plants might protect plants from the disease, improves growth and increase medicinally important secondary metabolites.

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