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Original Article

Monitoring of Defense Enzymes (Phenylalanine Ammonia Lyase and Peroxidase) in *Magnaporthe oryzae* Infected Leaves after Treatment with Green Synthesized Silver Nanoparticles

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ABSTRACT

Objectives: In this study, we focus on the amount of the two defence enzymes phenylalanine ammonia-lyase (PAL) and peroxidase (POX) expressed with regard to the treatment of green synthesized silver nanoparticles. **Materials and Methods:** The leaf blades were infected with Fungal spores and silver nanoparticles by spraying, and following infection, we estimated the enzyme activity of PAL and POX. The PAL activity is considered by using cinnamic acid as a standard. **Results:** Our results showed both the PAL and POX enzymes were found to be elevated on infection with spores, and on treatment, the activity was reduced in both cases. There was a significant elevation of PAL (8.937±0.55) in infected leaves, which could be due to infection and on treatment, the enzyme activity was reduced which possibly could be because of the antifungal activity of the synthesised nanoparticles. The POX levels exceeded the control by 417.86%, 807.14% and 921.43% respectively for infected, treatment with AgNPs and treatment with positive control. This confirms the role of the peroxide enzyme in disease control and resistance. **Conclusion:** This enzyme activity study to our knowledge is the first report done on the infected rice plants with *Magnaporthe oryzae* and on treatment with silver nanoparticles. This could pave the way to understanding the role of these enzymes in defence activity.

Keywords: *Magnaporthe oryzae*, PAL, POX, Silver Nanoparticles, Defense enzymes.

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INTRODUCTION

Magnaporthe oryzae causes critical diseases called rice blast. In tropical Asian nations like India and China, the output of rice decreased by up to 69%.[1] The use of chemical fungicides to treat rice blast disease posed a serious risk to the environment and to the general public's health. Earlier, chemical fungicides were employed to treat soil-borne fungal diseases, but these chemicals are still present in the agriculture ecosystem today, where they harm helpful bacteria and allow plant pathogens to become resistant to them.[2] As a result, we must discover a different biocontrol strategy that can control plant diseases without endangering human health or the environment.[3] One of the best options recently as a reliable and safe clarification for sustainable agriculture is biological control. An environmentally beneficial way for lowering the possibility of resistance to selection pressure is biocontrol.[4]

Recent findings reported that nanoparticles can be an eco-friendly solution for fungal diseases and many plant extracts are used for metal ion reduction to produce metal nanoparticles. among different metals, silver has shown very good antifungal activity.

There are passive and active defence mechanisms which are being used to defend themselves during pathogen attack.[5] In the passive defence mechanism, antimicrobial molecules are vital compounds while in active barriers pathogenesis-related proteins, hypersensitive response, phytoalexins, the production of reactive oxygen species (ROS) and lignification.[6] The resistance or susceptible reaction to fungal infection is determined by the influence of defence responses by the plants during pathogen infection. In plant fungal infection, penetration may induce different plant cell defence responses during different infection stages.[7] We should say that the defence enzymes are vital to the plant during infection.[8] The activity of Peroxidase (POX) and Phenylalanine Ammonia-Lyase (PAL) are observed to be crucial in the wheat defence mechanism. We can say changes in PAL and

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Peroxidase (POX) activity happens in wheat plant in different stages of infection, which play an important role in resistance to the pathogen.[9] reported that the activity of PAL and peroxidase increased during the development of the leaf infection. The peroxidase activity also increased immediately after increasing the PAL activity.[9]

Researchers observed that different signalling compounds play a critical role in the plant's resistance to the pathogen and a significant role of Peroxidase (POX) and Phenylalanine ammonia-lyase (PAL) in the phenylpropanoid (PP) pathway that help in establishing the plant resistance against leaf infection.[10]

To catalyse the oxidoreduction of a variety of substrates, POX (Class III plant peroxidase; EC 1.11.1.7) uses hydrogen peroxide.[11] phytoalexin synthesis, production of ROS, cross-linking of cell wall proteins, hydrogen peroxide scavenging, antifungal activity of the POX itself,[12] auxin metabolism and thickening of the xylem wall, are a few of the reports that show POXs play vital roles in resistance to pathogens.

Some peroxidase (POX) genes are activated by pathogens such as viroids, bacteria, viruses and fungi.[13-15] It belongs to the pathogenesis-related (PR) protein-9 family as a result. In rice blast fungus inoculation boosted POX activity and *Xanthomonas oryzae* pv. *oryzae* infection, a bacterial leaf blight disease, promoted the expression of POX8.1 and POX22.3 genes, especially in a resistant rice cultivar. Additionally, expression of the POX gene in rice, tomato, horseradish and tobacco plants is increased by wounding.[15,16]

In our study, we designed to estimate the effect of enzymes in resistance mechanisms and to understand the expression of PAL and POX following *M.oryzae* infection in Rice plants.

MATERIALS AND METHODS

Materials

Garlic cloves were purchased from local market stores and the outer covering was removed and sun-dried for 4–5 days so as to remove moisture completely. Dried cloves were pulverized to a fine powder and stored in a dry container for further use. Silver nitrate ($AgNO_3$) was purchased from HiMedia Ltd., Bangalore. All the reagents used in the study were of analytical and molecular grade. For the experiment, all of the solutions were made fresh with deionized water and maintained in the dark to avoid any photochemical reactions.

Micro-organisms

Antifungal activity of green synthesized silver nanoparticles was studied against *Magnaporthe oryzae*. Fungal cultures were isolated from the infected paddy seeds. Oatmeal agar was used to maintain the strain. The fungal culture grown in the laboratory was rechecked for the specific conidia to confirm the strain.

Green Synthesis of silver nanoparticles

AgNPs were synthesized by reduction of silver nitrate with garlic extract by the following method. About 200ml deionized water was added to 20 g of garlic powder prepared in the previous section. The contents were then kept on a magnetic stirrer for one hour at 40^0C and filtered with Whatman No.1 filter paper and finally centrifuged at 10000 rpm for 20 min. The filtrate was then evaporated in a rotary evaporator and about 50mg of extract was then suspended in 40 ml of deionized water. An equal volume of 0.5 mM $AgNO_3$ was added to the suspension and incubated for 2 days at 50-70^0C with constant stirring on the magnetic stirrer. The pH of the solution was maintained at 10. Following characterization (not shown in this paper), we assessed the influence of the synthesized nanoparticles on enzymatic activity.

Samples

Infected leaves were considered as control, infected leaves sprayed with nanoparticles (FNP), infected leaves sprayed with nanoparticles (3 days treatment) (3FNP), infected leaves sprayed with Bavistin, positive control (FB), infected leaves sprayed with Bavistin (3 days treatment) (3FB).

Phenylalanine ammonia-lyase (PAL) activity

Preparation of cinnamic acid standards

Dissolved 2mg of cinnamic acid in 100 ml of distilled water to prepare a stock solution. Then prepared five different concentrations that were 0.2 µl, 0.4 µl, 0.6 µl, 0.8 µl and 1.0µl. and then we observed the absorbance at 270nm.

Extraction of PAL

Phenylalanine ammonia-lyase extraction is done according to Ballester.[17] About 40mg of acetone powder formed in the previous section was added with 1.5mL of sodium borate buffer (100mM; pH 8.0) containing 20mM of β-mercaptoethanol. The extract was homogenised for about 2min. The contents were then centrifuged at 13,000 rpm. The supernatant obtained was further purified and salting out was done with 60% ammonium sulphate. The protein fraction was incubated on ice for 30min and centrifuged at 13,000 rpm for 30 min. Pellet obtained was resuspended in 1.5mL of 100 mM sodium borate buffer (pH 8.0). The remaining suspension was used for the determination of PAL activity. The assay mixture contained 0.66mL of enzyme solution and 0.2mL of 0.1 M l phenylalanine, in a total volume of 2 mL. The control, instead of l-phenylalanine, contained distilled water. We incubated the sample for 2hr at 40°C. The production of cinnamate for 120 min at 40°C measured by the absorbance change at 270 nm determined the PAL activity.

Peroxidase activity

The activity of peroxidase was assayed by a spectrophotometric method as described by SIGMA Aldrich. This spectrophotometric

rate determination is based on the following reaction: One unit of peroxidase is said to form 1mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C. This unit is equivalent to ~18μM units per minute at 25°C.

$$H_2O_2 + \text{Pyrogallol (donor)} \xrightarrow{peroxidase} 2H_2O_2 + \text{Purpurgallin (oxidized donor)}$$

Leaf samples

The leaves after infection, were excised after two days for the assay. Leaf samples were collected for about 6 days in total. Rice leaf sheaths (0.5gm) (both treated and control) were homogenized in 10ml of 0.1M phosphate buffer (pH 7.0) using a pre-chilled porcelain mortar and pestled at 4°C. The homogenate was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used for assay of peroxidase.

In brief, the sample components were added as mentioned in the below Figure 1. Final Assay Concentrations – In a 3.00 mL reaction mix, the final concentrations are 14mM potassium phosphate, 0.027% (v/v) hydrogen peroxide, 0.5% (w/v) pyrogallol, and 0.45–0.75 unit peroxidase. To each sample tube labelled, 0.32ml of phosphate buffer (100mM Potassium Phosphate Buffer, pH 6.0 at 20°C), and 0.16ml of peroxide solution (0.50%) were added and mixed well. Then 0.32m of pyrogallol solution (5%) was added to the contents and added with Millipore water to make up to 2.9ml. Sterile distilled water serves as blank. The contents are thoroughly mixed by inverting the tubes gently and equilibrating at 20°C in a suitably thermostatted spectrophotometer for ~10 min. To all the sample tubes, 0.1ml of peroxide working solution was added and 0.1ml of phosphate buffer was added to the blank.

The contents following mixing were then observed for absorbance (A_{420}) at 420nm for 3 min. Enzyme activity was calculated using the following formula.

$$\text{Units/ml enzyme} = \frac{\Delta A420/20\text{secTest sample} - \Delta A420/20\text{secBlank}) * 3 * df}{12 * 2.1}$$

Where 3 = Volume (in milliliters) of assay; d_f = Dilution factor; 12.0 = Extinction coefficient of 1 mg/mL of Purpurogallin at 420 nm (determined internally); 0.1 = Volume (in milliliters) of enzyme used.

RESULTS

The green synthesis AgNPs from cloves were used in the study. The synthesized GAgNps were found to be of the size ranging from 30-60nm. UV spectra, FTIR analysis was done to check the quality of the particles. SEM analysis revealed the green synthesized AgNP were found to be of 30-60nm size (Results not shown in this paper. Green synthesis manuscript was corresponded for publication to journal).

Peroxidase Activity

Our results showed that the PAL enzyme activity in the control was 2.471 ±0.12. The PAL activity was seen to be elevated (8.937±0.55) in infected leaves. These enhanced results indicate the PAL enzyme activity which could be induced by infection. The treatment with nanoparticles reduced the enzyme activity which possibly could be due to the antifungal activity of the synthesised nanoparticles. The concentrations of the samples were calculated from the cinnamic standard graph (Y=10.167x, R^2=0.9941).

There is a significant effect observed on treatment with nanoparticles when compared to positive control Bavastin.

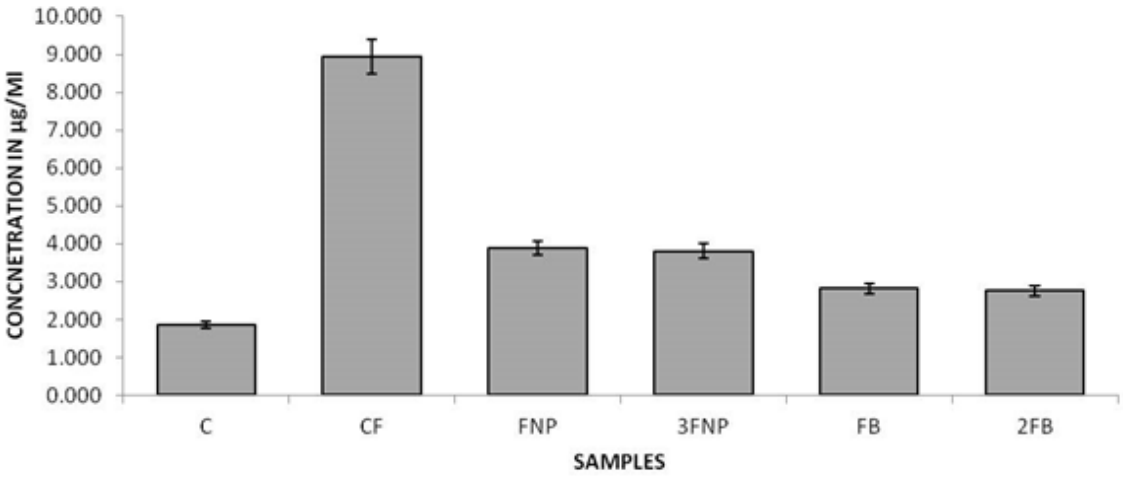

Figure 1: Graph showing the concentration of enzyme obtained after extraction. C: control (Healthy leaf; without infection); CF: Control with infection; FNP: infected leaves sprayed with nanoparticles. 3FNP: infected leaves sprayed with nanoparticles (3 days treatment); FB: infected leaves sprayed with Bavastin; 3FB: infected leaves sprayed with Bavastin (3 days treatment). All the values are average of triplicates and the error bars indicate the standard error ($p \leq 0.05$).

However, there is no significance observed between immediate treatment and after 3 days of treatment.

A two-way ANOVA between the samples and the number of days was conducted to compare the PAL enzyme activity. There was a significant effect on the PAL activity between the sample and also of the number of days remembered at the $p<0.05$ level. The significant effect of the samples on the PAL activity was reported to be very high [F (3, 3) = 17.91, p = 2E-07]. There was also a significant effect but a very small number of days on the PAL activity [F (1, 3) = 0.0057, p = 0.0425].

Peroxidase activity

The peroxide activity was found to be enhanced for two days and thereafter seems to be stabilized. But the treatment showed a significant enhancement in the enzyme activity depicting its role in disease resistance. The enzyme activity was found to be 4.52± 1.05 and 8.74± 0.21at 1 and 2 days respectively for treatment with nanoparticles. This was significant when compared to a positive control (5.16± 0.21 and 9.12± 0.2 for 1 and 2 days respectively).

Since the 2-day activity was found to be elevated abnormally, the values are highlighted throughout the study. The enzyme levels exceeded the control by 417.86%, 807.14% and 921.43% respectively for infected, treatment with AgNPs and treatment with positive control. This confirms the role of the peroxide enzyme in disease control and resistance.

The enzymatic activity was modified by the application of nanoparticles as can be from the graph below. The control showed the lowest activity of peroxide, while the treatment of *Magnaporthe oryzae* enhanced the activity confirming the role of peroxide in disease resistance. The treatment of nanoparticles was consistent, showing the highest peroxide activity when compared to positive control (Figure 2).

The enzyme levels exceeded the control by 417.86%, 807.14% and 921.43% respectively for infected, treatment with AgNPs and treatment with positive control. This confirms the role of the peroxide enzyme in disease control and resistance.

DISCUSSION

Numerous pathogenic bacteria produce the rice illnesses that significantly reduce yields across the globe. Since rice is a staple diet for more than half of the world's population, such losses are significant. Rice has become a model for researching plant-microbe interactions of monocotyledons due to the intensive investigation of the molecular interactions among rice and the fungus disease *Magnaporthe oryzae* during the past two decades. Our study confirmed of the possible role of the defense enzymes on infection with their abnormal elevation levels. Our green synthesized nanopartciles were found to be significantly effecting the enzymatic activity confirming of the potential role of antifungal activity.

PAL enzyme activity, is seen as a variation in absorbance at 270nm, in the infected leaves of rice. In almost all plants, phenylpropanoid pathways generate critical metabolites like flavonoids, isoflavonoids, lignins, anthocyanins and other antimicrobial compounds used for defending themselves.[18] PAL is an enzyme which converts L-phenylalanine to trans-cinnamic acid by removing ammonia. This trans-cinnamic acid is again used as a precursor for synthesizing flavonoids.[19] Enhanced PAL activity is seen as a defence reaction in the plants as to protect against the pathogens.[20]

Similar results were seen according to[21] wherein they observed enhanced enzyme activity in the treatment of the rice sheath infected with *Alternaria solani* with selenium and Copper nanoparticles. The peroxide levels were found to be surpassed the control and fungal pathogen by 438% and 436%, respectively.

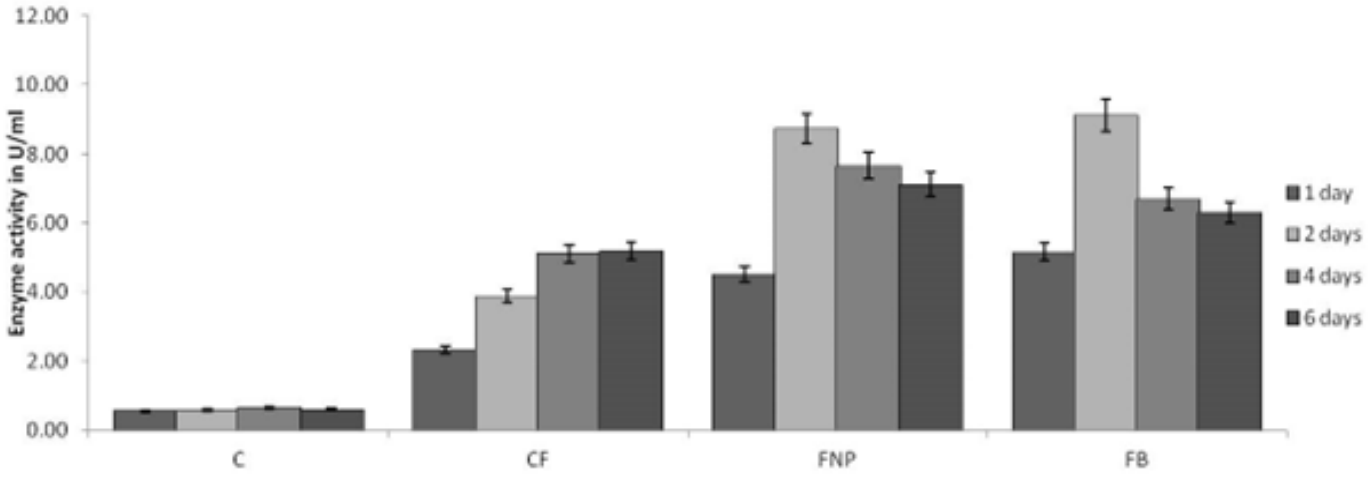

Figure 2: Graph showing the changes in peroxidase activity in rice leaf sheath tissues on infection with fungus and treatment with nanoparticles. The activity was determined colourimetrically. All the values are average triplicates and represented as value ± SD. C: control without infection; CF: Control with infection; FNP: infected and treated with AgNPs; FB: infected and treated with positive control.

Increased PO activity, lignin deposition, an increase in phenolic content, and the induction of PR proteins are frequently linked to induced resistance in plants.[22] Plants' lignification, suberization, polymerization of hydroxy proline-rich glycoproteins, control of cell wall elongation, wound healing, and resistance to pathogens are all regulated by peroxidases.[23]

Similar to our findings, rice leaf sheaths showed a significant increase in PO activity one day after treatment with OA. This increase peaked two days later, at which point a two-fold increase in PO activity was detected in comparison to the control plants. This increased PO in rice leaves treated with OA may have a role in the production of lignin, which in turn may have facilitated the development of disease resistance.[24]

Our results are also in consistent to those of Susanto U (2012), where a significant reduction in the PAL enzyme activity was observed rice varieties on infection with *Xanthomonas oryzae* infection. This confirms the role of PAL against the infection rates.

CONCLUSION

The results of the current study revealed that plant immunity to Rice Blast was enhanced as a result of multi-component signalling network engagement. The current investigation confirmed the crucial roles that phenylalanine ammonia-lyase (PAL) and peroxidase (POD) played in the phenylpropanoid (PP) pathway in developing rice's resistance to *M. oryzae* infection. The *M. oryzae* infection had an impact on the enzyme activities, which were further amplified during resistance expression. And after treatment, we discovered that both enzyme levels were lower than they were under control.

By boosting its activity in the cells after *M. oryzae* infection, PAL helps rice types with their resistance mechanism. Real-time PCR assays are being used to conduct more expression investigations, which will help to clarify the function of these enzymes in defensive activities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

POX: Peroxidase; **PAL:** Phenylalanine Ammonia-Lyase; $\mathbf{d_f}$**:** Dilution factor.

SUMMARY

The rice/*M. oryzae* pathosystems especially have been the subject of considerable investigation over the past two decades and have developed into molecular models for studies on plant-microbe interactions. Plants do express certain defenseive enzymes in response to pathogens like fungus and bacteria. These enzymes play a critical role in protecting the host against harmful pathoegns. Among such enzymes PAL and Peroxidase stand first to provide an innate immunity to the host. On infection, we found these enzyme loads were abnormally elevated confirming of their role in defense. In addition we found our green synthesized nanopartciles could alter the enzyme activity suggesting of the potential role of antifungal activity.

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