

# Enhanced antioxidant system reprogrammes oxidative stress through hypersensitive response in leaves of rice plant challenged with *Aspergillus* species

Padmanabh Dwivedi\*, Asha Kumari and Bansh Narayan Singh

Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221005, India

\*Corresponding author: pdwivedi25@rediffmail.com

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## Abstract

Nitric Oxide (NO) is involved in diverse physiological processes in plants. As a developmental regulator, it promotes germination, leaf extension and root growth, and also delays leaf senescence and fruit maturation. As modulator of disease resistance, it triggers hypersensitive cell death or Hypersensitivity Response (HR) and activates the expression of several defense genes. This wide variety of effects reflects the basic signaling mechanisms that are utilized by virtually all living organisms. The ubiquity of NO reactions as well as the finding that the biochemical and molecular mechanisms underlying many physiological processes are well conserved between diverse species has opened the exploration of NO chemistry in different organisms. Experiments were conducted in Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University to study the NO mediated HR during interaction of *Aspergillus* sp. with rice plants. The pathogen (*Aspergillus* sp) was infiltrated in leaves of rice plants (*Oryza sativa*), and the responses were observed after 6, 12, 24 and 48 h of infection. The experiment was laid out in Factorial Complete Randomized Design which consisted of 8 treatments, 3 replications. Different biochemical parameters like Nitrite content, Proline, H<sub>2</sub>O<sub>2</sub>, SOD, Ascorbate Peroxidase (APX), Total Phenol Content (TPC), Phenyl ammonia lyase (PAL) were measured. PAL and TPC measures the quantity of Systemic Acquired Resistance (SAR) developed in plants against pathogen attack. Among the above mentioned parameters except nitrite, proline and H<sub>2</sub>O<sub>2</sub>, all other parameters showed maximum activity at 48 h treatment. The results indicate that HR was developed during rice-*Aspergillus* interaction through enhanced anti-oxidant system, possibly mediated through NO, thereby restricting pathogen's spread after 12 to 48 h of infection of rice leaves with *Aspergillus* sp.

## Highlights

- Nitrite content regulates hypersensitive response during rice plants – *Aspergillus* sp. interaction, indicating for participation of nitric oxide signaling in the process
- Increased anti-oxidant system and Systemic Acquired Resistance facilitated development of hypersensitive response in rice plants against *Aspergillus* infection
- The results have significant bearing on disease management in rice against fungal pathogen. It also demonstrated, for the first time, the participation of anti-oxidants system in plants during hypersensitive response triggered by nitric oxide through enhanced nitrite production in rice plants upon interaction with plant pathogen, thus facilitating defense response against the pathogen

**Keywords:** *Aspergillus*, infiltration, griess reagent, hypersensitive response, nitric oxide, *oryza sativa*, pathogen

Rice is a monocotyledonous angiosperm. The genus, to which it belongs, *Oryza*, contains more

than 20 species, only two of which are referred to as cultivated rice as *Oryza sativa*, cultivated in



South-East Asian countries and Japan, and *Oryza glaberrima* cultivated in West Africa. Rice was originally cultivated in tropical Asia, the oldest record dating 5000 years BC, but then extended also to temperate regions. More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population lives. With the world population estimated to increase to about 8.2 billion in the year 2030, the global rice demand will rise to about 765 million tonnes, or 533 million tons of milled rice (FAO 2002). For almost three decades since the Green Revolution, the rice yield growth rate was approximately 2.5% per year. During the 1990s, however, this has decreased to 1.1%. The reason for this decline is attributed partly to the fact that it has served as a host for a number of diseases and insect-pests, 54 in the temperate zone and about 500 in tropical countries. Of the major diseases, 45 are fungal, 10 bacterial, 15 viral (Ou 1985) while 75 are insect-pests and nematodes. Realizing the economic losses caused by them, efforts have been directed to understand the genetic basis of resistance and susceptibility.

But, in nature, plants have unique quality to develop defense mechanism against biotic and abiotic stress i.e. through Hypersensitivity Response (HR) or oxidative burst (Apel and Hirt 2004). The HR apparently protects plant against attack by various pathogens and might enhance plant immunity in different ways. Nitric oxide (NO) has been considered as potential signaling molecule involved in triggering defense against various pathogenic attack in plants (Dwivedi and Choudhury 2012)). It has been demonstrated that NO can be produced by nitrite via plasma membrane bound nitrite: NO reductase either by plant roots or cells (Stohr *et al.* 2001; Dwivedi, and Choudhury 2012) and through mitochondrial electron transport (Planchet *et al.* 2005; Dwivedi and Singh); it participates in HR mediated defense response. Both reactive nitrogen species and reactive oxygen species are primarily involved in signal cascade leading to HR (Delledonne *et al.* 1998; Durner and Klessig 1999; Klessig *et al.* 2000; Neill *et al.* 2002; Lamotte *et al.* 2004; Wendehenne, Durner and Klessig 2004).

Oxidative burst situation is created by invasion of pathogens generated by highly reactive oxygen species (ROS). Depending on level, ROS may be beneficial or harmful. At low level it works as

secondary messenger (Williams and others 2011), and at high level, decreases seed viability, root growth, and causes leaf abscission and desiccation, breakdown of lipids, proteins, carbohydrates, chloroplasts or carotenoids (Philosoph-Hadas *et al.* 1994). To remove from system, plants have acquired antioxidant machinery system such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), glutathione reductase (GR), glutathione-S-transferase (GST), peroxidases (POX) as well as certain non-enzymatic plant antioxidants such as carotenoids, tocopherols, phenolics, ascorbate, glutathione, flavonoids and proline (Nizamuddin 1987; Wojtaszek 1997; Kang and Saltveit 2002; Jain *et al.* 2011,12). Each plant has its own general strategies to improve self immunity under stresses (El-Zahaby and others 1995). Susceptibility of pathogens to a plant depends on biotic factors to generate cellular antioxidant capability and signal cascade like systemic acquired resistance (SAR).

*Aspergillus* species is a cosmopolitan, highly aerobic fungus found in almost all oxygen-rich environments. It is commonly found in soil and causes serious disease not only in animals but highly affected in number of crops (Klich 2002). *Aspergillus* is commonly powdery masses of brown color on the lower surface. In grains and legumes, infection area is often appearing downy or powdery. Hyphae are rapidly grown and produce a thread like mycelium. After successful establishment of mycelium, it secretes degradative enzymes or proteins which can break down complex nutrients. Therefore, keeping the overall mechanisms of plant defense responses against this pathogen, experiments were conducted to understand various events involved in intra-cellular defense mechanisms in rice plants against *Apergillus* infection.

## Materials and methods

### *Isolation, multiplication and suspension culture preparation of pathogen*

An isolate of *Aspergillus* species was obtained from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi.(Courtesy, Dr. B K Sarma, Associate Professor) The culture of *Aspergillus* was maintained on potato dextrose agar (PDA, MO96; Himedia, Mumbai, India) by transferring



the mycelia with the help of an inoculation needle and incubated at  $28 \pm 2^\circ\text{C}$ . After two weeks, spores carefully transferred into 10 mM  $\text{MgCl}_2$  solution. Density of suspension culture was kept around 1.41  $\text{OD}_{600}$ .  $\text{MgCl}_2$  solution without spore culture served as control for each experiment.

### Experimental designing

Rice seeds (*Oryza sativa* L.) was procured from Department of Genetic and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Surface sterilization of seeds was performed with the help of 0.1%  $\text{HgCl}_2$  for 30 sec and washed three times with distilled water. Seeds (15-20) were sown in plastic pots (20x20 cm) containing sterilized soil:sand:FYM (2:1:1). Whole set up was transferred into plant growth chamber provided with 8h/16h (light/dark) condition,  $24^\circ\text{C}$  temperature, 75% relative humidity for 21 days.

### Biochemical measurements

#### Estimation of nitrite content

Nitrite content was estimated by Griess reagent as per method described by Planchet *et al.* (2005), which is based on two-step diazotization reaction. Leaf samples (100 mg) was added in 300  $\mu\text{l}$  Griess reagent (5% phosphoric acid containing 0.1% NED in 1% sulfanilamide), 50  $\mu\text{l}$  0.5 M zinc acetate and volume made up to 3 mL with distilled water, incubated in dark for 30 min at room temperature. Then the absorbance of the reaction mixture was measured at 540 nm.

#### Qualitative assessment of $\text{H}_2\text{O}_2$ production

HR lesions developed in leaves were kept in 2 mL of DAB staining solution as described by Thordal-Christensen *et al.* (1997). Tubes were kept in shaker for 4-5 h at 80-100 rpm in dark at  $25^\circ\text{C}$ . De-chlorophyllization was performed by placing lesion samples in bleaching solution (ethanol/acetic acid/glycerol; 3:1:1) for 15 min at  $90^\circ\text{C}$  in water bath. Replaced the bleaching solution with fresh bleaching solution and allowed to stand for 30 min. At last leaf samples were placed on slides and observed under microscope.

#### Hydrogen peroxide estimation

Hydrogen peroxide was determined by the method described by Mukherjee and Choudhary (1983). Leaf

samples (100 mg) were homogenized in 10 mL cold acetone and filtered through Whatman No.1 filter paper. Reaction mixture containing filtrate and 4 mL of titanium reagent was added followed by 5 mL of concentrated ammonium solution to precipitate peroxide- titanium complex. The contents were centrifuged for 5 min at 10,000 rpm and precipitate dissolved in 10 mL 2 N  $\text{H}_2\text{SO}_4$ . It was centrifuged to remove undissolved material and absorbance was recorded at 415 nm against blank (2 N  $\text{H}_2\text{SO}_4$ ).

#### Estimation of Ascorbate Peroxidase (APX) activity

Ascorbate peroxidase activity was estimated in the first fully expanded leaf of rice according to the method proposed by Nakano and Asada (1981). It is based on the decrease in absorbance at 290 nm as ascorbate is oxidized. The reaction mixture for the peroxidase contained 0.1 mL of 100 mM potassium phosphate buffer (pH 7.5), 0.1 mL of 1.5 mM  $\text{H}_2\text{O}_2$ , 0.40 mL of 3 mM ascorbic acid, 0.1 mL of 3 mM EDTA and 1 mL of enzyme extract in a total volume of 3 mL. In blank, ascorbic acid was not added. The reaction was started by adding the enzyme and decrease in absorbance was recorded 30 sec after this addition at 430 nm.

#### Estimation of superoxide dismutase (SOD) activity

SOD activity was estimated by the method proposed by Dhindsa *et al.* (1981). Leaf samples (100 mg) homogenized in 1 mL of 0.1 M potassium phosphate buffer on ice chilled mortar and pestle and centrifuged at 16000 rpm for 20 min at  $4^\circ\text{C}$ . Three mL of the reaction mixture containing 0.1 mL of 1.5 M sodium carbonate, 0.2 mL of 200 mM methionine, 0.1 mL of 2.25 mM NBT, 0.1 mL of 3 mM EDTA, 1.5 mL of 100 mM potassium phosphate buffer, 1 mL of DW and 0.1 mL of enzyme extract were taken in test tubes in replicates from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 mL riboflavin (60  $\mu\text{M}$ ) and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes by black cloth. Tubes without enzyme extract developed maximum colour. A non irradiated complete mixture that did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme was taken as the quantity of enzyme which reduced the absorbance



reading of sample to 50 per cent in comparison to the tubes lacking enzyme.

### Determination of total phenol content (TPC)

Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu method as described earlier (Wolfe *et al.* 2003). An aliquot (100  $\mu$ l) of the extracts was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10, v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was recorded against reagent blank at 765 nm using the Simadzu UV-VIS spectrophotometer. Samples of extract were evaluated at a final concentration of 0.1 mg/mL. Total phenolic content was expressed as mg per gram gallic acid equivalent.

### Determination of Phenyl Ammonia Lyase (PAL)

HR samples (0.5 mg) were crushed with borate buffer, 1.3 mL of distilled water were mixed with the extracted sample then finally reaction was started by adding 1mL phenylalanine solution and incubated for 30-60 min at 32°C, according to the method described by Brueske (1980). Reaction was stopped by using 0.5 mL of 1 mL TCA. Absorbance was taken at 290 nm. Standard curve was prepared using trans-cinnamic acid.

### Free proline content

HR samples (0.5 g) were homogenized in 5 mL of sulphosalicylic acid (3 per cent) using pre-chilled mortar and pestle and centrifuged at 10,000 g for 10 min. Residue was again extracted twice with 5 mL of 3% aqueous sulphosalicylic acid. All the supernatant fractions were pooled and final volume was made to 15 mL. Two mL of the extract was taken in the test tube and 2 mL each of glacial acetic acid and ninhydrin reagent were added. The reaction mixture was boiled in water bath at 100°C for 30 min till brick red colour developed. After cooling the reaction mixture, 5 mL of toluene was added and then transferred to separating funnel and the absorbance was read at 520 nm using spectrophotometer against toluene blank, as described by Bates *et al.* (1973). Concentration of proline in the samples was estimated by referring to a standard curve of proline.

### Statistical analysis

Values from different experiments represented mean and SEM of at least three replications from each experiment. The analysis of variance was carried out separately for each experiment for various time intervals according to the procedure of Factorial Completely Randomized Design.

## Results and discussion

### *Effect of Nitrite content in hypersensitive response development*

Plants have several cellular defense mechanisms to protect themselves against pathogens. In the present experiments, we studied the interaction of different molecules involved in plant defense mechanisms mediated through hypersensitive response. HR is the first line of immune response developed by plant after elicitation of any foreign agent, serving as innate immune local response. During infiltration of *Aspergillus* pathogen in the rice leaves, the HR developed (Figure 1A); in the HR lesion samples production of nitrite continuously increased up to 24 h, but maximum nitrite content obtained after the leaves were challenged with pathogen was 0.030 ( $\mu$ mol  $g^{-1}h^{-1}FW$ ) as compared to control 0.028 ( $\mu$ mol  $g^{-1}h^{-1}FW$ ) (Table 1). It is not governed by a single molecule, but has complex molecular interactions of nitrogen generating species and reactive oxygen generating species (ROS).

Nitrite production is an indication of NO emission during infection which gets converted into a toxic compound peroxynitrite (ONOO) after interaction with ROS generating molecules, which causes local cell death (Delledonne *et al.* 2001; Radi *et al.* 2005; Gerardo Ferrer-Sueta and Rafael Radi, 2009). In this experiment, production of  $H_2O_2$  along with nitrite content was increased. Qualitative and quantitative analysis of hydrogen peroxide production was estimated by DAB staining and a procedure described in materials and methods, respectively. Reddish brown stain by DAB was more prominent in *Aspergillus* infiltrated rice leaves as compared to control (Figure 1 B & C). Quantitative measurement of  $H_2O_2$  content indicated maximum production after 24 h of infection with pathogen (0.003  $\mu$ mol  $g^{-1}FW$ ), but HR in control rice leaves (0.001  $\mu$ mol  $g^{-1}FW$ ) was not observed (Table 2).



Production of  $H_2O_2$  leads to HR mediated program cell death (Dat *et al.* 2003). If they are not removed from system cause cell damage to host by non-host pathogens. Therefore, several antioxidant s are involved in removing ROS molecules (Vacca *et al.* 2004; Baxter *et al.* 2014). In this experiment, APX activity in HR lesion samples continuously increased up to 48 h (Table 3). The maximum APX content in control was 0.0061 (nmol Ascorbic acid oxidized  $min^{-1} mg^{-1}$  protein) while in those challenged with *Aspergillus* (HR samples) was 0.0093 (nmol Ascorbic acid oxidized  $min^{-1} mg^{-1}$  protein). Another antioxidant scavenger enzyme, SOD was produced continuously in rice leaves after infiltration of *Aspergillus*. In infiltrated rice leaves, the maximum 11.0 (EU  $g^{-1}$  FW) SOD activity while 7.6 (EU  $g^{-1}$  FW) in control were observed at 48 h (Table 4). They work together to promote scavenging of free radicals. A similar result was reported in cultivars of *Phaseolus vulgaris* treated with *Uromyces phaseoli* (Buonaurio *et al.* 1987). Increase in antioxidant enzymatic machinery may lead to improvement in stress tolerance (Murgia *et al.* 2004; Lee *et al.* 2007). Infection of *Erysiphe graminis* f. sp. *hordei* leads to increased antioxidant metabolism in barley (El-Zahaby *et al.* 1995).

Phenol production is a positive indicator of plant's resistance which plays key role as antimicrobial defense arsenal of plants (Shoresh and Harman 2008; Abo-Elyousr *et al.* 2009). In this experiment, total phenol content (TPC) increased significantly higher compared to un-infiltrated (10 mM  $MgCl_2$ ) after being challenged with *Aspergillus* (Table 5). Small quantity of phenol changes was also detected in control i.e. 1.3 (mM gallic acid $^{-1}g^{-1}$  FW); however, in leaf samples infiltrated with pathogen, it was slightly higher i.e., 1.7 (mM gallic acid $^{-1}g^{-1}$  FW) at 48 h. Moreover, induction of phenol content is correlated with induced PAL activity, which is a key enzyme of phenyl- propanoid biosynthesis pathway, in the synthesis of phenols or phytoalexins. These are involved in reinforcement of plant cell wall and salicylic acid signaling molecules (Nicholson and Hammerschmidt, 1992; Wen *et al.* 2005). In the present study, maximum PAL activity was detected in the rice plants infiltrated with *Aspergillus* compared to control at the end of 48 h infection

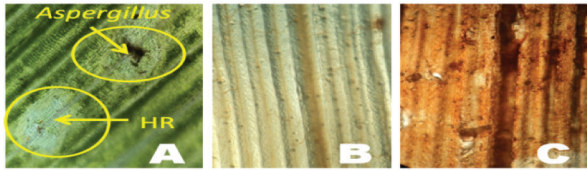
(Table 6). After infiltration, PAL activity increased at all the time intervals and maximum (25.0  $\mu M$  TCA  $g^{-1}$ FW) was recorded at 48 h; however, in control it was less (15.5  $\mu M$  TCA  $g^{-1}$ FW) as compared to infiltrated regions of leaves. Increased TPC and PAL activity in this experiment may, thus, be correlated with accelerated defense response against fungal pathogen, *Aspergillus* species in rice plants.

Apart from activation of defense molecules as described above, some other molecules are involved in stress tolerance such as proline, flavonoids and terpenes. Proline is an amino acid which takes part in maintaining membrane stability. It can increase several folds under stress conditions. In the present study, proline accumulation increased at early stage i.e., 6 h thereafter declined up to 48 h in *Aspergillus* infiltrated and non- infiltrated rice leaves. Maximum proline accumulation in *Aspergillus* infiltrated rice leaves was found to be 9.39 (mg  $g^{-1}$  fresh weight) while minimum in control 4.14 (mg  $g^{-1}$  fresh weight) at 6 h (Table 7). A similar result was observed in *Arabidopsis* challenged with races of *Pseudomonas syringae* pv tomato (Pst), which triggered proline accumulation indicating an association with development of the hypersensitive response (Fabro *et al.* 2004). It may be correlated with induced defense response along with TPC and PAL activation.

**Table 1:** Analysis of nitrite content ( $\mu mol g^{-1}h^{-1}$ FW) in HR induced by *Aspergillus* species in rice leaves (hpi)

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> sp. infiltration	Source	SEM	CD
6	0.011	0.018	Treatment	0.000	0.0003
12	0.013	0.023	D (time)	0.000	0.0001
24	0.028	0.030	T X D	0.000	0.0004
48	0.025	0.028			

Mean and standard error of mean (SEM) were calculated for estimation of nitrite from leaf area of 0.75  $cm^2$ . Rice leaves were infiltrated with the *Aspergillus* sp. Data were collected from three leaves in each experiment. Data were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.



**Fig. 1:** Infiltration of suspension culture of *Aspergillus* in rice leaves. Black spot in upper yellow circle indicated the spores and HR- like symptom developed in lower circle (A).  $H_2O_2$  staining with DAB in rice leaves (C) but were absent in control (B), upon rice leaf infiltration with *Aspergillus*. After 24 h, the *Aspergillus* induced HR response. Brown color indicates the  $H_2O_2$  productions whereas faint color developed indicated for less production of  $H_2O_2$ . Similar results were obtained in three independent experiments.

**Table 2:** Analysis of  $H_2O_2$  content ( $\mu\text{mol g}^{-1}$  FW) in rice plants after infiltration of fungal pathogen *Aspergillus* sp. at different time intervals (hpi).

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	0.000	0.001	Treatment	0.0006	0.0025
12	0.001	0.001	D (time)	0.0001	0.0004
24	0.001	0.003	T X D	0.0003	0.0012
48	0.001	0.001			

Mean and standard error of mean (SEM) were calculated for estimation of  $H_2O_2$  content from a leaf area of  $0.75 \text{ cm}^2$ . Rice leaves were infiltrated with the *Aspergillus* sp. Data were collected from three leaves in each experiment and were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

**Table 3:** APX activity ( $\text{nmol Ascorbic acid oxidized min}^{-1} \text{ mg}^{-1}$  protein) in rice plants after infiltration of fungal pathogen *Aspergillus* sp. at different time intervals (hpi)

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	0.0029	0.0029	Treatment	0.0001	0.0005
12	0.0034	0.0035	D (time)	0.0001	0.0006
24	0.0044	0.0057	T X D	0.0001	0.0005
48	0.0061	0.0093			

Mean and standard error of mean (SEM) were calculated. Rice leaves were infiltrated with the *Aspergillus* sp. Data were collected from three leaves in each experiment. Data were significantly

different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

**Table 4:** SOD activity ( $\text{EU g}^{-1}\text{FW}$ ) in rice plants after infiltration of fungal pathogen *Aspergillus* sp. at different time intervals (hpi)

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	6.3	8.1	Treatment	0.070	0.287
12	6.7	9.1	D (time)	0.035	0.144
24	7.1	10.1	T X D	0.098	0.406
48	7.6	11.0			

Mean and standard error of mean (SEM) were calculated. Rice leaves were infiltrated with the *Aspergillus* sp. Data were collected from three leaves in each experiment and data were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

**Table 5:** Total phenol content ( $\text{mM gallic acid}^{-1}\text{g}^{-1}\text{FW}$ ) in rice plants after infiltration of fungal pathogen *Aspergillus* sp. at different time intervals (hpi)

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	0.7	1.3	Treatment	0.008	0.032
12	0.8	1.3	D (time)	0.004	0.016
24	1.0	1.5	T X D	0.011	0.045
48	1.3	1.7			

Mean and standard error of mean (SEM) were calculated. Rice leaves were infiltrated with the *Aspergillus* sp. Data were collected from three leaves in each experiment. Data were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

**Table 6:** PAL activity ( $\mu\text{M TCA g}^{-1}\text{fresh weight}$ ) in rice plants after infiltration of fungal pathogen *Aspergillus* sp. at different time intervals (hpi)

Time (hpi)	Control (10 mM $MgCl_2$ )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	7.9	17.4	Treatment	0.053	0.220
12	11.4	21.8	D (time)	0.027	0.110
24	13.7	23.0	T X D	0.075	0.311
48	15.5	25.0			



Mean and standard error of mean (SEM) were calculated. Rice leaves were infiltrated with the *Aspergillus sp.* Data were collected from three leaves in each experiment. The data were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

**Table 7:** Free proline content (mg g<sup>-1</sup> fresh weight) in rice plants after infiltration of fungal pathogen *Aspergillus sp.* at different time intervals (hpi)

Time (hpi)	Control (10 mM MgCl <sub>2</sub> )	<i>Aspergillus</i> spp. infiltration	Source	SEM	CD
6	4.143	9.390	Treatment	0.003	0.013
12	3.346	9.116	D (time)	0.002	0.007
24	1.863	2.979	T X D	0.005	0.019
48	1.284	1.370			

Mean and standard error of mean (SEM) were calculated. Rice leaves were infiltrated with the *Aspergillus sp.* Data were collected from three leaves in each experiment. The data were significantly different at  $P < 0.05$  in all experiments. Hpi, hours post infection.

## Conclusion

In the present study we investigated that host plant has intra-cellular defense mechanisms to counteract the oxidative stress induced by pathogens. Enhanced antioxidant enzymatic system, TPC content and PAL activity mitigated oxidative burst, mediated through increased nitrite content, thus possibly involving nitric oxide signaling; this explained the protection from pathogen stress. Additional investigations related to nitric oxide mediated HR in plant-fungal pathogen interaction need to be strengthened.

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