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# Utilization of fungal biocontrol agents against rice sheath blight disease provides insight into their role in plant defense responses

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Biotic and abiotic factors have an effect on rice production all around the world. Diseases are regarded as major restrictions among the biotic stressors, and rice sheath blight (*Rhizoctonia solani* Kühn) is one of the most calamitous diseases that significantly damage the crop. Lately, biocontrol of fungal plant pathogens has appeared as an appealing approach. The present investigation was undertaken to evaluate different biocontrol agents like *Talaromyces flavus*, *Chaetomium globosum*, *Pseudomonas fluorescens* and *Aspergillus niger* against sheath blight disease. Prior to sowing, seeds were bioprimed with each isolate and sown in the nursery. After 21 days, seedlings were transplanted *in-vivo* and were inoculated with a virulent isolate of *Rhizoctonia solani* at maximum tillering stage. Observations on biochemical parameters and gene expression studies were carried out at 24, 48, 72, and 96 hpi. Enzymatic activity *viz*., chitinase, β-1,3-glucanase, catalase, and PAL was observed maximum in *Chaetomium globosum*. PR-genes viz.,IPT, BrD, HmPr, AMP, AldD, NIC and LisH showed up-regulation at 96 hpi. *Chaetomium globosum* had the highest yield, maximum number of tillers with least relative lesion height (RLH%) as compared to other treatments. However, results indicated biocontrol agents are helpful and they induce multitude of defence responses against *R. solani* in rice.

**Keywords**: Biocontrol agents, PAL, PB-1121, *Rhizoctonia solani*, Sheath blight

Rice, the world's most significant food crop, has been produced throughout South Asia for over 6000 years. Rice is currently the staple diet of about four billion people, or half of the world's population[1]. Various biotic and abiotic factors have an impact on rice production around the world. Diseases are one of the most important biotic pressures on rice production, with rice sheath blight, caused by *Rhizoctonia solani* Kühn, being one of the most serious diseases capable of causing major crop damage in areas where intensive agriculture is practise[2].This disease is also called as "snake skin disease," "mosaic foot stalk," and "rotten foot stalk" because to its distinctive symptoms[3-4]. Rice sheath blight is a serious damaging disease that causes quality degradation and up to a 50% of yield loss each year around the world[5-7]. In India, annual losses due to sheath blight (ShB) are estimated to be 10%[8]. For the management of this disease, chemical control strategies are already in use, but a strategy for eco-friendly compounds with enhanced disease control potential is critical[9].

*Rhizoctonia solani* is a phytopathogenic fungus that infects a wide variety of plants [10]. This disease affects a lot of plant groups, including the Asteraceae, Brassicaceae, Fabaceae, Poaceae, and Solanaceae, as well as several woody ornamental plants and forest trees[11-14]. Biological management of fungal plant diseases has recently emerged as an appealing and feasible option which helps disease management as well as improves the plant growth characters also. The principal antagonistic microorganisms against numerous plant pathogens are *Chaetomium* spp., *Aspergillus* spp., *Pseudomonas* spp., and *Talaromyces* spp., which were used as biological control for the pathogens such as *Gaeumannomyces graminis* var. *tritici*[15], *Fusarium oxysporum*[16], *Sclerotium rolfsii*[17], *Verticillium dahliae*[17], *Verticillium albo-atrum*[18], *Rhizoctonia solani*[19] and *Sclerotinia sclerotiorum*[20-21]. *Chaetomium globosum* has the potential to be a biocontrol agent for a variety of seed and soil-borne diseases[22]. In general, bio-control agents work through competition, mycoparasitism, antibiosis, or a combination of these mechanisms to stop the growth of bacteria and fungus[23]. *Pseudomonas fluorescens* has emerged as a promising biocontrol agent for plant

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disease suppression by protecting seeds and roots against fungal infection. They have been shown to promote plant development and lower the severity of numerous fungal infections[24-25]. *Aspergillus niger* has an antagonistic relationship with *R. solani*. It lowers the occurrence of disease[26].

Plant defence induction is a novel biological strategy for controlling plant disease. Many biocontrol agents are being studied for their ability to induce plant systemic resistance to pathogenic fungus. There are definite signs of systemic defense-related enzyme activity in the soybean, tomato, and *Arabidopsis thaliana* plants. These enzymes include phenylalanine ammonia lyase (PAL)[27-30].

Most hyperparasitic biocontrol agents produce enzymes like chitinases and glucanases. Plant pathogens can use specific methods to defend themselves against cell wall destroying enzymes. In fact, *R. solani* secretes a wide variety of effectors, enzymes that are active on carbohydrates, and various kinds of secondary metabolites that have an impact on host immunity[31-33]. Plants have numerous layers of defences against pathogen attacks to counterbalance the effects of pathogenicity factors in plant diseases. Pathogenicity-related proteins such as β-1,3-glucanase, and chitinase plays a critical role in fungal-pathogen infection defence[34]. β-1,3-glucanase hydrolyzes β-1,3-glucans, which are found in most fungal cell walls, whereas chitin is a significant component of fungal cell walls that is hydrolyzed by the chitinase enzyme[35].

Here, we tested four different biocontrol agents: *Chaetomium globosum* (Cg 2), *Pseudomonas fluorescens* (DTPF-3), *Aspergillus niger* (ITCC no. 7790), and *Talaromyces flavus* (Tf2) against *Rhizoctonia solani* inoculated plants to see how they affected overall plant growth and development, as well as defence response to the phytopathogenic fungus *R. solani*. As a result, numerous plant growth parameters were examined in the presence of pathogen and biocontrol agents. In addition, the activities of defense-related enzymes were characterised, and the expression of distinct pathogenicity-related genes in plants was studied.

## Materials and Methods

### Location of experiment

All the experimental studies were carried out at ICAR-Indian Agricultural Research Institute (IARI), Pusa campus, New Delhi at latitude of 28.08°N and longitude of 77.12°E, the height above mean sea level being 228.61 metres (750 feet).

### Fungal isolates, rice genotypes taken for the study

Highly virulent isolate of *R. solani* collected from various rice growing areas in Uttar Pradesh, India. R-359 (ON383512) was selected for inoculation. Slants of Potato Dextrose Agar (PDA) were used to culture and preserve isolates, and they were kept at 4°C. For present study, the susceptible rice cultivar Pusa Basmati-1121 was obtained from Division of Genetics, IARI, New Delhi.

### Biocontrol agents and seed treatment

Biocontrol agents *Talaromyces flavus* Tf2[36], *Chaetomium globosum* Cg2[37], *Aspergillus niger*[38], and *Pseudomonas fluorescence* DTPF-3[39] were taken from Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi. Spore suspensions of biocontrol agents prepared separately and passed through double-layered muslin fabric and concentration of spores were adjusted to $1 \times 10^7$ spores $mL^{-1}$ (*Talaromyces flavus, Chaetomium globosum* and *Aspergillus niger*) and $1 \times 10^8$ spores $mL^{-1}$ (*Pseudomonas fluorescence*). Seeds were dipped in biocontrol suspension for 24 h. Treated seeds were further sown in nursery bed prepared in glasshouse. Twenty-one days old nursery of the "Pusa Basmati 1121" treated with different biocontrol agents were uprooted carefully and transplanted at experimental field of ICAR-IARI. The normal agronomical practices with recommended doses of fertilizers (N: $P_2O_5$: $K_2O$: 120: 60: 60 kg $ha^{-1}$) were followed. Plots were kept weeds free through manual weeding. Seeds soaked only in water were treated as control.

### Mass multiplication and inoculation of *Rhizoctonia solani*

Forty five days old transplanted plants (Maximum tillering stage) were inoculated with virulent isolate of *Rhizoctonia solani*. For which Typha (*Typha angustifolia*) stems were cut into 4-5 cm lengths, lightly filled 3/4$^{th}$ of a conical flask (500 mL), after that it was autoclaved for 15 min at temperature of 121°C, 15psi pressure. The Typha pieces were inoculated with fresh R-359 (ON383512) culture in a laminar air flow hood and incubated for one week at 26±1°C in BOD. As an inoculum, Typha pieces fully colonized with *Rhizoctonia solani* were utilised[40] in the rice genotype PB 1121. Inoculum (3 to 4 Typha bits) was inserted at the base of the tillers, and thread was used to bind the typha bits to the tillers, making contact between both the mycelium and rice plants. Three duplicates were kept for each isolates, as well as a control (three inoculated hills per pot were

considered as one replication and 3 replicates per isolates were evaluated).

**Phenotypic evaluation of rice genotypes in field**

Different parameters such as plant height, lesion height, numbers of tillers and grain yield were recorded for sheath blight disease severity. The relative lesion height (RLH) was calculated by using the following formula:

$$\text{RLH} = \frac{\text{Lesion height}}{\text{Plant height}} \times 100$$

**Sample collection**

For biochemical and real time PCR based analysis infected rice plants were collected at 24, 48, 72, and 96 h post inoculation. These samples were taken in triplicate and transferred to liquid $N_2$ after being covered in aluminium foil. After that, the samples were kept at −80°C for future examination.

**Biochemical analysis**

***Enzyme extract preparation***

In liquid $N_2$, one gram of each sample was crushed to a fine powder. At 4°C, 3 mL of sodium acetate buffer (50 mM, pH 5.2) was mixed with this tissue powder. At 4°C, the enzyme-buffer combination was centrifuged for 10 min at 10000 rpm. The supernatant was placed into a sterile tube and utilised in enzyme detection experiments.

***Chitinase activity assay***

The reduction of 3, 5-dinitrosalicylic acid in the presence of the amino-sugar N-acetyl-D-glucosamine (NAG) was used to determine chitinase enzyme activity. Chitin enzymatic hydrolysis releases NAG. The protocol for assaying chitinase was modified somewhat from that published by[41].

In 1 mL of enzyme extract, we added 1 mL of 10% wet colloidal chitin (in 0.2 M sodium phosphate buffer, pH 6.5). The reaction mixture was then incubated at 50°C for 30 min. After that, we added 1 mL of 1% NaOH to the reaction mixture. To stop the enzymatic action, NaOH is utilized. The mixture was then centrifuged at 4°C for 10 min at 7500 rpm. In a sterile tube, the supernatant was collected. One mL of 1 percent 3, 5-dinitrosalicylic acid was combined with 1 Ml of supernatant. 3, 5-dinitrosalicylic acid was dissolved in 30 percent sodium potassium tartrate and 2 M NaOH to make a solution. For 5 min, these tubes were immersed in boiling water. The reaction mixture was then diluted 1:10 with water after the tubes were allowed to cool to room temperature. At 535 nm, the absorbance of chitinase activity was measured. The amount of enzyme necessary to create 1 µmol of NAG in 1h was defined as a chitinase activity unit.

Four grams of chitin (Himedia) was added to 60 mL concentrated hydrochloric acid and stirred continuously at 4°C to make a colloidal chitin suspension. It was left at 4°C overnight after mixing. After 24 h, 500 mL of ice cold 95 percent ethanol was added without shaking and incubated at 4°C overnight. Chitin is precipitated by ice cold 95 percent ethanol. The ethanol was then removed by centrifuging the mixture at 10000 g for 10 min at 4°C. To remove the remaining ethanol, 10 mL ice cold sterile water was added to wash and centrifuged at 10000 g for 10 min at 4°C temperature. The washing process was repeated twice more before being autoclaved.

***β-1,3-Glucanase activity assay***

Abeles and Forrence's approach was used to detect β-1,3-glucanase activity. Laminarin was utilised as the substrate, and dinitrosalicylic was employed as the reagent, to measure the synthesis of reduced sugar[42]. In a sterile tube, 1 mL crude enzyme was added to 1 mL 2 percent laminarin solution. Tubes were incubated at 50°C for 60 min. Heating was used to incorporate laminarin into the reaction mixture. 3 mL dinitrosalicylic reagent was added to the reaction mixture after it had been heated. The tubes were heated at 100°C for another 5 min. The dinitrosalicylic reagent is used to halt the process. One gram NaOH, 0.2 g phenol, 0.05 g sodium sulphite, 18.2 g sodium potassium tartarate (Rochelle salt), and 1 g 3,5-dinitrosalicyclic acid were combined in a 100 mL distilled water solution to make the dinitrosalicyclic reagent. The reaction mixture was then diluted 1:10 with water after the tubes were allowed to cool to room temperature. At 500 nm, the absorbance was measured.

***Phenylalanine Ammonia Lyase (PAL) activity assay***

The enzyme activity of phenylalanine ammonia lyase (PAL) was measured using Mori *et al.*[43] technique. At 37°C, L-phenylalanine transforms to cinnamic acid. 0.4 mL Tris-HCl buffer (100 mM, pH 8.8) and 0.2 mL phenylalanine (40 mM) were added to 0.2 mL enzyme extract to make the reaction mixture. In place of enzyme extract, 30 µL extraction buffer (50 mM sodium acetate buffer, pH5.2) was employed as a control. The absorbance of the samples

was measured at 290 nm, with readings obtained every 30 sec for 5 min after the reaction began. PAL activity was measured in millimoles of cinnamic acid per milligrams of protein.

***Catalase activity assay***

The activity of the catalase enzyme was measured using the Bailly *et al.* technique[44]. In the tube, we added 40 µL of crude enzyme and 3 mL of 0.068 M potassium phosphate buffer (pH-6.8). The reaction began after 40 µL of 10 mM H2O2 was added to the tube, and the optical density was measured at 240 nm. The change in absorbance at 240 nm in the linear phase of the slope ($\Delta 240$ $min^{-1}$ $mg^{-1}$ protein) was used to measure catalase activity.

***$H_2O_2$ content estimation***

Alexieva *et al.* method[45] was used to determine the hydrogen peroxide concentration in plant leaves after reacting with KI, $H_2O_2$ was detected spectrophotometrically. 0.5 mL 0.1 percent trichloroacetic acid (TCA) leaf extract supernatant, 0.5 mL 100 mM K-phosphate buffer, and 2 mL reagent (1 M KI *w/v* in fresh double-distilled water) made up the reaction mixture. In the absence of leaf extract, the blank probe comprised of 0.1% TCA. The reaction was developed in the dark for 1 h, and the absorbance was measured at 390 nm. A standard curve with known $H_2O_2$ values was used to calculate the amount of hydrogen peroxide.

***Malondialdehyde (MDA) content estimation***

The amount of malondialdehyde (MDA), a result of unsaturated fatty acid peroxidation, can be used to quantify lipid peroxidation. The MDA concentration (mg $g^{-1}$ fresh weight) was calculated using Li *et al.* technique[46]. A total of 0.5 g of fresh leaves was homogenised in 10 mL of 10% trichloroacetic acid (TCA) and centrifuged at 12000 g for 10 min. An aliquot of 2 mL of the supernatant was then added to two millilitres of 0.6 percent thiobarbituric acid in 10% TCA. The combination was cooked for 30 min in boiling water before being immediately chilled in an ice bath. After centrifugation at 10000g for 10 min, the absorbance of the supernatant was measured at 450, 532, and 600 nm. The MDA concentration, which was expressed as nmol $g^{-1}$ DW, was estimated from the formula: C (nmol $mL^{-1}$) = 6.45 ($A_{532}$-$A_{600}$)-0.56($A_{450}$).

***Protein assay***

The protein content in the extract was estimated by the Bradford method using bovine serum albumin as standard.

***Primer designing***

For primer synthesis, the Primer3 Plus software (www.bioinformatics.nl/primer3plus) was utilised. The software was loaded with nucleotide sequences, qRT-PCR was selected as the reaction type, and a product size of 150–250 bp was chosen. The forward and reverse primers with the fewest variances in several parameters such as GC content, melting temperature, and self-complementarity were chosen, and specificity was validated using the NCBI nucleotide blast. The primers were designed in India by GCC Biotech Pvt. Ltd. For the experiment, the stock primers were diluted in a 1:10 ratio and maintained at −20°C. A total of seven pathogenicity-related primers were employed (Table 1[47-49]). For the amplification of *Rhizoctonia solani* cDNA, the housekeeping gene GADPH was included[47].

**Gene expression studies**

***RNA isolation***

Plants were watered two times a day, and inoculation was done in the evening. At 24, 48, 72, and 96 h after inoculation, the inoculated plants were harvested. The plants were labelled and covered in aluminium foil before being preserved at −80°C. The total RNA from infected rice sheaths was obtained using the Trizol technique (Invitrogen). All laboratory materials such as eppendorf tubes, spatulas, microcentrifuge tubes, pestle, and mortar were sterilised with DEPC (Diethyl pyro carbonate) treatment @ 0.1 percent for the inactivation of RNAse enzymes. These materials were autoclaved for 15 min at 121°C and 15psi. Infected Pusa Basmati 1121 were homogenised with liquid $N_2$ in a pre-cooled mortar and pestle. To thoroughly dissociate the nucleoprotein complex, the fine powder (approximately 200 mg) was incubated in a 1.5 mL eppendorf tube with 1 mL Trizol reagent for 5 min at room temperature. For phase separation, the tubes were filled with chloroform (0.2 mL per 1 mL Trizol). The tubes were vigorously shaken by hand for 15 sec, allowed to cool for 2 to 3 min before being centrifuged at 12000 g for 15 min at 4°C. The aqueous phase was transferred to fresh tubes, pre-chilled with 0.5 mL isopropanol, and then centrifuged at 12000 g for 10 min at 4°C. By removing the supernatant solution from the tubes, the RNA pellet was maintained. The pellets were centrifuged at 7500 g for 5 min in 4°C after being rinsed with 1 mL of 75 percent ethanol. After removing the wash, the pellets were dried in air for 10 min. The pellets were dissolved in 30 µL of DEPC-treated water and kept at

Table 1 —*Rhizoctonia solani* primers used for qRT-PCR analysis[47-49]

| S. No. | Genes name | Primer name | Primer sequence |
|---|---|---|---|
| 1 | Inorganic phosphate transporter (IPT) | IPT-F | TCTTGACCACGGGGAGATAC |
| | | IPT-R | GGATGTCCAGAGCAAACCAT |
| 2 | Bromodomain containing protein (BrD) | BrD-F | ATGCGCTCATTGAGGATTTC |
| | | BrD-R | CGGTAATCTACGCCCACACT |
| 3 | Aldehyde dehydrogenase (AldD) | AldD-F | AATGGGCAGACTTGGATGAG |
| | | AldD-R | GGAGGGTTATCGACAAACGA |
| 4 | AMP bindingdomain (AMP) | AMP-F | CAAGAAGGAGCTCGAAGTGG |
| | | AMP-R | GGCGGCCACTTTTATTGTTA |
| 5 | Hemeperoxidase (HmPr) | HmPr-F | GCTTGGAACAAGACGAGGAG |
| | | HmPr-R | TTGAGAATAGGGCGAGGAGA |
| 6 | Lissencephaly1 homolog (LisH) | LisH-F | GAGCTTACCTTGCGACCTTG |
| | | LisH-R | ATGTAGATGTGCGGTGTGGA |
| 7 | Nuclear poreprotein, Nic96 (NIC) | NIC-F | TGCGTTCTAACAGAGGCGTA |
| | | NIC-R | ATTCGTGGACTCAACCAAGC |
| 8 | Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) | GAPDH-F | CAAAGTGACCAGCGACTTGA |
| | | GAPDH-R | GCCTTGTCCTTGTCAGTGAAG |

−80°C. The RNA was quantified using a Nano Drop-2000 Spectrophotometer (Thermo Fisher Scientific, USA).

***cDNA synthesis***

The Verso cDNA synthesis kit was used to transform the RNA template into cDNA according to the manufacturer guidelines (Verso cDNA, Thermo scientific, USA). OligodT primers were used to create first strand cDNA. In the C1000TM thermal cycler, reverse transcription settings consist of one cycle at 42°C for 30 min and one cycle of inactivation at 95°C for 2 min (Bio rad, USA). The cDNA was kept at a temperature of −20°C.

***qRT-PCR analysis***

The qRT- PCR samples were set up in triplicates, with a non-template control. For the reactions, 96-well qRT-PCR plates (Axygen, Inc., California) were used. Real-time PCR was performed using the Quant Studio 12K Flex real-time PCR machine (Applied Biosystems by Life Technologies, USA). The target gene amplification was monitored using DyNAmo flash SYBR (2X) green mix dye (Thermo Scientific, USA). To ensure the fluorescent dye's efficacy, the SYBR green mix dye was utilised in a dark environment. The total reaction volume was 20 µL, with 10 µL of SYBR, 1 µL of forward and reverse primers, 1µL of template cDNA, and 7 µL of nuclease-free water. In qRT-PCR setup, Initial denaturation at 95°C for 10 min is followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 51 to 54°C for 30 sec (different for each primer pair), extension at 72°C for 1 min, then default dissociation curve analysis to assess amplicon specificity. The target gene expression was quantified using the $2^{-\Delta\Delta Ct}$ technique using the reference gene GAPDH as a control[50]. The pathogenicity gene average Ct values were subtracted from the average Ct values for the GAPDH gene. ΔCt values are determined by substracting values. After then, ΔΔCt values for each treatment were determined by subtracting their individual ΔCt values from the ΔCt value of the negative control (calibrator). ΔΔCt was calculated using the formula below:

$$\Delta\Delta Ct = (C_{t\ target} - C_{t\ reference})_{Time\ x} - (C_{t\ target} - C_{t\ reference})_{Time\ 0}$$

where, Time x = any time point and Time 0 = 1x expression of the target gene normalized to reference gene.

Finally, using the formula $2^{-\Delta\Delta Ct}$, the fold change expression data was derived. Standard deviation calculated from three technical replicates on each of three independent biological studies is represented by error bars.

**Statistical analysis**

Statistical analysis were done for all replicated samples by using OPSTAT software. Replications were maintained in a completely randomized design and all experiments were repeated twice and critical difference was calculated. For biochemical and gene

Table 2 — Phenotypic evaluation of rice genotypes treated with biocontrol agents

| S.no. | Treatments | Lesion height (cm) | Plant height (cm) | Relative lesion height (%) | Number of tillers | Yield (g/m$^2$) |
|---|---|---|---|---|---|---|
| 1 | *Chaetomium globosum* | 60.62 | 89.87 | 68.26 | 37.5 | 497 |
| 2 | *Aspergillus niger* | 63.87 | 84 | 75.75 | 26.25 | 458 |
| 3 | *Talaromyces flavus* | 63.12 | 76.87 | 82.45 | 31.62 | 464 |
| 4 | *Pseudomonas fluorescens* | 67.5 | 88.75 | 77.89 | 23.12 | 436 |
| 5 | Control (*R-359*) | 84.25 | 96 | 87.78 | 24.62 | 404 |
| 6 | Critical difference | 6.66 | 5.65 | 9.05 | 4.92 | 6.14 |

expression studies data are expressed as mean of 3 independent biological and 3 technical replications with ± standard deviations.

## Results

**Evaluation of rice genotypes for sheath blight disease severity:**

The disease severity of the *Rhizoctonia solani* virulent isolate (*R-359*) in Pusa Basmati 1121 was assessed using Relative Lesion Height (RLH %), plant height, lesion length, number of tillers, and grain yield. Different biocontrol agents, including as *Talaromyces flavus*, *Chaetomium globosum, Pseudomonas fluorescens*, and *Aspergillus niger*, showed significant differences in Pusa Basmati 1121 (Table 2). Inoculated control had the highest relative lesion height (87.78 cm), while treatment *Chaetomium globosum* had the lowest relative lesion height (68.26 cm). Treatment *Chaetomium globosum* had the highest average number of tillers (37.5), whereas, treatment *Pseudomonas fluorescens* had the lowest average number of tillers (23.12). The highest yield was found in *Chaetomium globosum* (497g/m$^2$), whereas the lowest yield was observed (436 g/m$^2$) in plants treated with *Pseudomonas fluorescens*.

**Biochemical analysis**

N-acetylglucosamine polymer chitin is hydrolyzed by the chitinase enzyme. Chitinase enzyme lowers 3,5dinitrosalicyclic acid and forms a yellow colour product in the presence of N-acetylglucosamine polymer chitin[41]. Chitinase enzyme activity was measured in *Chaetomium globosum* treated plants at 48 hpi and *Pseudomonas fluorescens* treated plants at 72 hpi, with maximum enzyme activity (4.53 mol chitin min$^{-1}$ mg protein$^{-1}$) and minimum enzyme activity (0.030 mol chitin min$^{-1}$ mg protein$^{-1}$), respectively, (Fig 1A).

Laminarin is employed as a substrate for the glucanase enzyme in the β-1,3-glucanase enzyme assay, which yields reducing sugars. Dinitrosalicylic acid becomes a dark brown product when it combines with the generated reducing sugar[42]. At 48 hpi, *Talaromyces flavus* had the highest enzyme activity (0.19 µmol laminarin min$^{-1}$ mg protein$^{-1}$). At 24 hpi, plants treated with *Pseudomonas fluorescens* had the lowest enzyme activity (0.04 µmol laminarin min$^{-1}$ mg protein$^{-1}$) (Fig 1B).

The production of cinnamic acid at 290 nm was used to test the phenylalanine lyase (PAL) assay. L-phenylalanine is converted to cinnamic acid by PAL. At 48 hpi, *Chaetomium globosum* treated plants had higher constitutive PAL enzyme activity (0.70 µmol L-phenylalanine min$^{-1}$ mg protein$^{-1}$). At 72 hpi, plants treated with *Pseudomonas fluorescens* had the lowest enzyme activity (0.15 µmol L-phenylalanine min$^{-1}$ mg protein$^{-1}$) (Fig 1C).

According to Bailly *et al.*[44], catalase enzyme assays were performed. The catalase enzyme uses $H_2O_2$ as a substrate. At 96 hpi, *Chaetomium globosum* treated plants had a greater catalase enzyme activity (60.16 µmol $H_2O_2$ min$^{-1}$ mg protein$^{-1}$). At 72 hpi, plants treated with *Pseudomonas fluorescens* had the lowest enzyme activity (12.72 µmol $H_2O_2$ min$^{-1}$ mg protein$^{-1}$) (Fig 1D).

$H_2O_2$ level was highest at 72 hpi (16.06 µmol g$^{-1}$) in the infected control. At 48 hpi, the minimum $H_2O_2$ level in *Aspergillus niger* treatment (5.14 µmol g$^{-1}$) (Fig 1E).

Malondialdehyde (MDA) levels were lowest at 24 hpi, in *Chaetomium globosum* treated plants (0.28 nmol mL$^{-1}$). At 96 hpi, *Talaromyces flavus*treated plants had a higher MDA concentration (0.94 nmol mL$^{-1}$) (Fig 1F).

**Determination of total protein content**

The Bradford method was used to determine the protein concentration of various materials (Table 3).

**Relative expression analysis**

In Pusa Basmati 1121, the expression pattern of pathogenicity-related genes of *Rhizoctonia solani* varied depending on the time and biocontrol agents used. The inorganic phosphate transporter (IPT) was marginally elevated at 96 hpi compared to 72 hpi

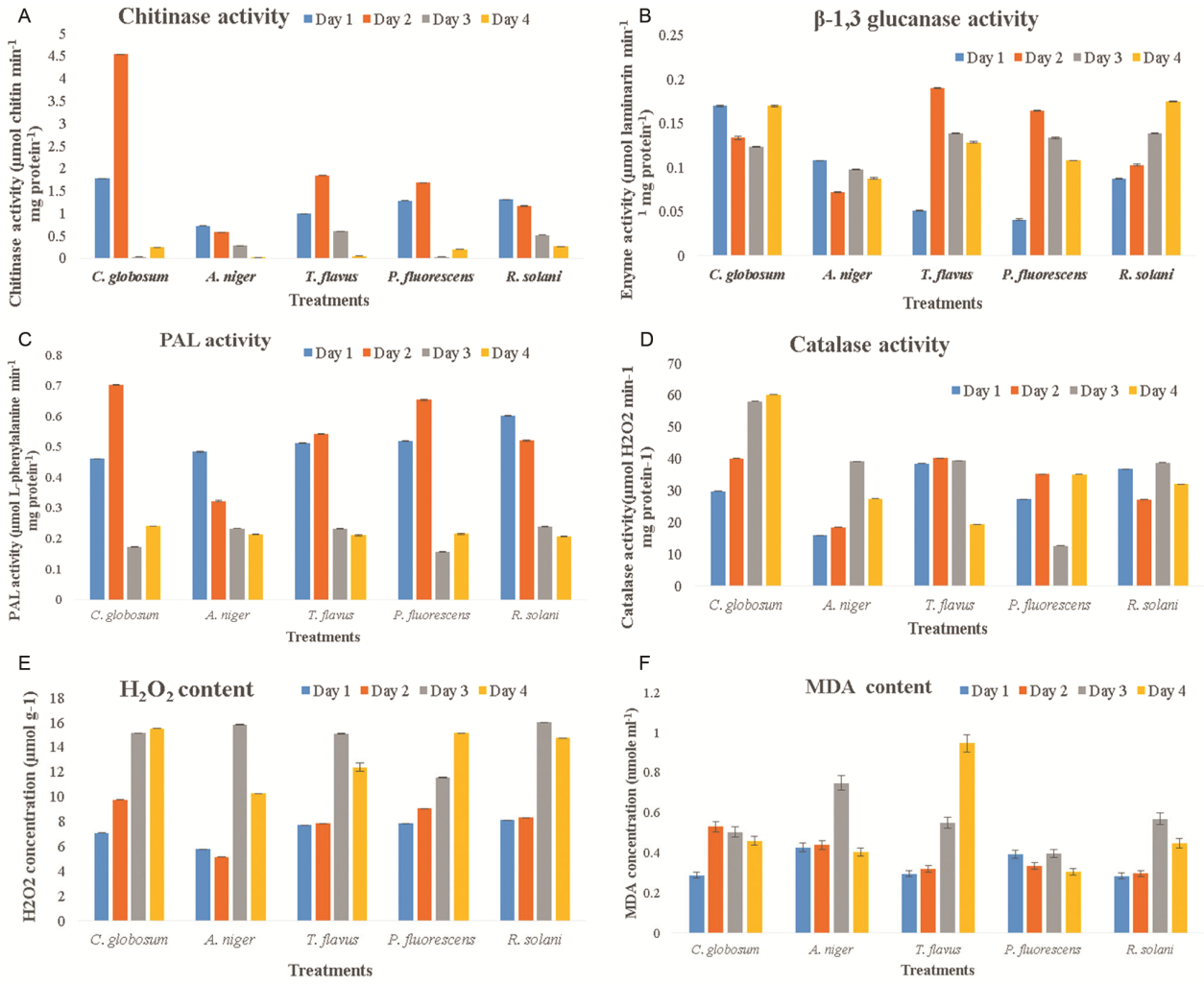


Fig 1 — Biochemical analysis of plant defence related enzymes in Pusa Basmati 1121 treated with biocontrol agents. (A)Chitinase activity, (B) β-1,3-glucanase activity, (C) Phenylalanine ammonia lyase activity, (D) Catalase activity, (E) Hydrogen peroxide ($H_2O_2$) content, and (F)Malondialdehyde (MDA) content

in all treatments, but relatively high expression at 96 hpi in the inoculated control (fold change 32.92) (Fig 2A).

In comparison to all other treatments, the expression pattern of Bromodomain containing protein (Brd) was highest in *Chaetomium globosum* at 96 hpi (fold change 39.36). At 72 hpi, all of the treatments had much lower expression, while at 96 hpi, they had significantly higher expression. In comparison to the other treatments, the inoculated control showed reduced expression (Fig 2B).

Inoculated control plants had significantly higher levels of heme peroxidase (HmPr) expression at 96 hpi (fold change 677.93) than other biocontrol treated plants. When compared to other treatments, the expression level of *Chaetomium globosum* was determined to be low at 96 hpi (fold change 8.33). In addition, when compared to 96 hpi, there was very little expressiveness at 72 hpi (Fig 2C).

When comparing the inoculation control (*Rhizoctonia solani*) to the remainder of the treatment, the gene AMP binding domain (AMP) expression was found to be highest at 96 hpi (fold change 22.28) in the inoculated control (*Rhizoctonia solani*). When compared to expression at 72 hpi, all treatments had higher expression at 96 hpi (Fig 2D).

In comparison to other treatments, the expression of the nuclear pore protein Nic96 (NIC) was highest in *Pseudomonas fluorescens* treated plants at 96 hpi (fold change 238.36). When compared to the inoculated control (fold change 108.23), *Talaromyces flavus* demonstrated considerable expression at 96 hpi (fold change 193.74). When compared to other treated plants, *Chaetomium globosum* showed the least

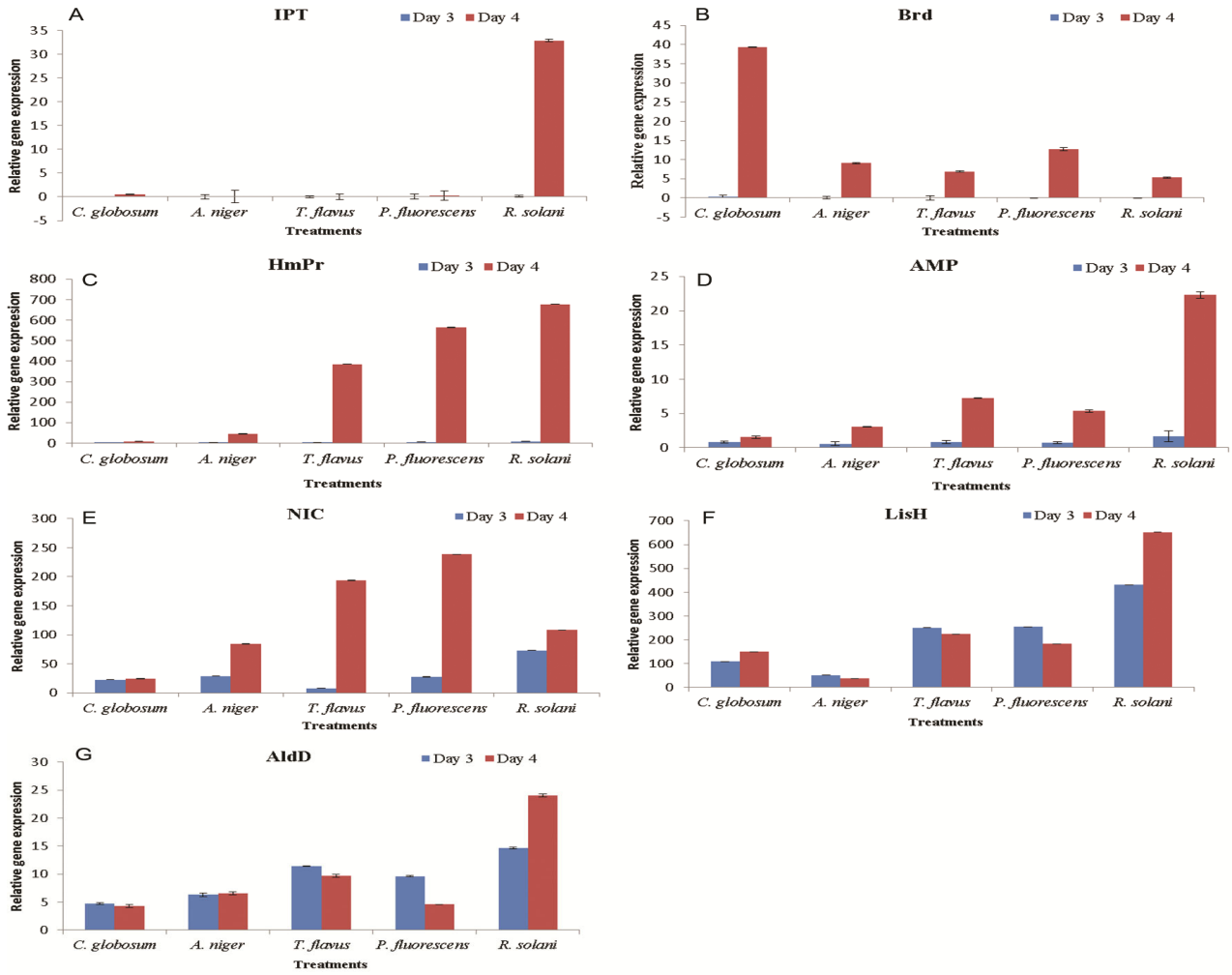


Fig 2 — *Rhizoctonia solani* gene expression study using qRT-PCR in PB-1121 treated with biocontrol agents at distinct hours of post-inoculation. (A) Inorganic phosphate transporter (IPT), (B) Bromodomain function (BrD), (C)Heme peroxidase (HmPr), (D) AMP binding domain (AMP), (E) Nuclear pore protein, Nic96 (NIC), (F) Lissencephaly-1 homolog (LisH), and (G) Aldehyde dehydrogenase (AldD)

expression at both time intervals, at 72 hpi (fold change 22.70) and 96 hpi (fold change 24.36). At both time intervals, the inoculated control showed good levels of expression and were upregulated (Fig 2E).

At 96 h after inoculation, the Lissencephaly-1 homolog (LisH) was expressed at its maximum level in the infected control (fold change 652.12). In comparison to 96 hpi, the expression was higher at 72 hpi with the treatments *Aspergillus niger* (fold change 51.66), *Talaromyces flavus* (fold change 251.07) and *Pseudomonas fluorescens* (fold change 254.05) (Fig 2F).

The expression of aldehyde dehydrogenase (AldD) in the inoculated control was substantially identical to that of the other treatments. *Chaetomium globosum* (fold change 4.7), *Talaromyces flavus* (fold change 11.42), and *Pseudomonas fluorescens* elevated expression levels at 96 hpi (fold change 9.64) (Fig 2G).

Table 3 — Protein concentration in mg $mL^{-1}$

| Treatments | Time Interval | | | |
|---|---|---|---|---|
| | 24 hpi | 48 hpi | 72 hpi | 96 hpi |
| Control(*R-359*) | 80.741 | 50.74 | 87.40 | 108.14 |
| *Chaetomium globosum* | 25.556 | 85.92 | 170.37 | 85.55 |
| *Aspergillus niger* | 37.03 | 40.37 | 62.22 | 138.88 |
| *Talaromyces flavus* | 52.22 | 30.00 | 91.11 | 173.33 |
| *Pseudomonas fluorescens* | 76.66 | 63.33 | 82.59 | 97.03 |
| Critical difference | 0.002 | 0.004 | 0.004 | 0.002 |

The inoculated control had a larger upregulation of pathogenicity-related genes, such as IPT, HmPr, AMP, Lish, and AldD, than the other treatments. At 96 hpi, genes such as Brd, IPT, HmPr, AMP, and NIC were found to be upregulated compared to 72 hpi.

## Discussion

*Rhizoctonia solani* Kühn is a soil-borne polyphagous pathogen that wreaks havoc on a wide range of vital staple crops. *Rhizoctonia solani* Kuhn produces rice sheath blight, which reduces yield dramatically by causing necrotic lesions on the leaves, sheaths, and grains.

Different biological controls were applied, which aided in reducing the disease. Due to the harmful impacts of pesticides and agrochemicals on ecosystems, biological controls have been demonstrated to be helpful in reducing plant diseases[51]. There are roughly 35 species of fungi and bacteria that have been utilised as biocontrol agents against various plant infections as an alternative to agrochemicals[52].

Biological controls, through physiological and biochemical changes, aid disease tolerance. In comparison to the inoculation control, rice plants treated with biocontrol agents had reduced relative lesion height and higher grain yield.

Chitinase is involved not only in plant defence, but also in plant development regulation[53], Plants generate enzymes that can break down pathogen cell walls, such as β-1,3-glucanase and chitinase[54]. These enzymes are major determinants of plant fungal disease resistance[55]. Hydrolytic enzymes act on fungal germlings shortly after pathogen penetration, weakening them and preventing disease development, whereas the pathogen may penetrate and colonise the tissue in susceptible hosts before PR proteins are induced to the needed level. The activity of chitinase, measured in mole of chitin $min^{-1}$ mg $protein^{-1}$, was shown to be lowest in the inoculated control at 48 hpi when compared to all other treatments. As a result, it is obvious that chitinase activity in plants treated with biocontrol agents was often higher than inoculation control plants. It has been shown that increasing chitinase activity in plants treated with biological controls reduced pathogen damage[56]. When compared to other time intervals, the enzyme activity were shown to be greater at 48 hpi. This could be related to plants' faster metabolic rate and increasing cells[57].

Pathogenic attacks, wounded tissue, UV radiation, heavy metal exposure, low temperature, and low amounts of nitrogen, phosphate, or ions are all factors that influence PAL activity[58]. The stress response PAL induction and activity can be used as a regulatory control. As a result, by tracking PAL activity, it is possible to forecast and support the defence response of various biological controls. Furthermore, PAL enzymes have a role in the synthesis of phenolic components that aid in the repair of plant wounds. Plants can use increased PAL activity as a defence mechanism to defend themselves against pathogen infection[59-62]. The inoculation control treatment had the lowest PAL enzyme activity, according to the results of the investigation. Infection treatment combined with biological controls, on the other hand, results in increased activity, which was highest with the treatment *Chaetomium globosum*, showing that wounding induced PAL enzyme activity. This finding confirmed that biological regulations have an effect on the expression of PAL, a defence system component.

Any derivative of oxygen molecules ($O_2$) that is deemed more reactive than $O_2$ itself is known to as a reactive oxygen species (ROS). Thus, ROS encompasses both free radicals like superoxide ($O_2$) and hydroxyl radicals (OH) as well as non-radicals such singlet oxygen ($1\ O_2$) and $H_2O_2$. Because of its relative stability, $H_2O_2$ has drawn a lot of attention as a signal molecule involved in the regulation of particular biological processes, including interactions between plants and pathogens. As opposed to all of the treatments investigated, the amount of $H_2O_2$ in the inoculation control increased slightly. As a result, all of the biological controls utilised to control the level of $H_2O_2$ had a very modest effect.

CAT is a tetramericheme-containing protein that is arguably one of the most well-known $H_2O_2$ breaking enzymes. While plants possess a variety of $H_2O_2$-metabolizing proteins, catalases seem to be highly active enzymes that catalyse a dismutase reaction and do not require cellular reductants. Catalases differ from many other peroxide-metabolizing enzymes in that they have a high selectivity for $H_2O_2$ but a low activity against organic peroxides[63]. In comparison to the other treatments, the treatment *Chaetomium globosum* had the highest catalase activity. As a result, it is obvious that *Chaetomium globosum* was determined to be the most effective biological control for metabolising peroxides such as $H_2O_2$.

To assess membrane damage in rice seedlings, lipid peroxidation in terms of malondialdehyde (MDA) was measured. When compared to the inoculation control and all other treatments, the treatment *Chaetomium globosum* had the lowest MDA content. To summarise, *Chaetomium globosum* was the most effective biological control for preventing rice seedling injury and yield loss.

*Rhizoctonia solani,* which infects rice, was studied in terms of gene expression. Most of the genes were dramatically raised in expression after rice infection in the susceptible variety Pusa Basmati 1121, and their expression altered over time.At 96 hpi, relative expression analysis of pathogenicity related genes such as inorganic phosphate transporter (IPT), heme peroxidase (HmPr), and AMP binding domain (AMP), all of which are involved in pathogen virulence, showed that inoculated control (32.92 folds IPT, 677.93 folds HmPr, and 22.28 folds AMP) was significantly higher than all other treatments. Heme peroxidase (HmPr) is a protein that helps to deactivate hydrogen peroxide. It aids pathogenicity inside the pathogen and immunity in the host. An acyl-activating enzyme (AAE) consensus motif is the AMP binding domain (AMP)[64] also expression level of these genes was upregulated at 96 hpi.

Bromodomain (BrD) expression was observed to be higher (39.36 times) in the *Chaetomium globosum* treatment, and it was also elevated at 96 hpi in all treatments. Many chromatin-associated proteins including nuclear histone acetyltransferases contain bromodomains. BrD-containing protein is an evolutionary conserved protein that plays a role in protein complex building via lysine acetylation and regulates gene transcription. This gene also plays a crucial role in cell proliferation. They predominantly interact with acetylated lysine[64].

Aldehyde dehydrogenase (AldD) is required for fungal pathogen development[65]. When compared to the other treated plants, the expression pattern of the AldD gene was increased in inoculated control plants at 72 hpi (14.72) and 96 hpi (24.06).

Nuclear pore protein (NIC) is thought to play a role in pathogenesis since it is required for cell proliferation and gene regulation[66-67]. When 96 hpi is compared to 72 hpi, the expression of this gene is elevated in all treatments. At 96 hpi, *Pseudomonas fluorescens* displayed the most expression (238.36).

Through dynein and microtubule-dependent mechanisms, Lissencephaly-1 homolog (LisH) is crucial for mitotic spindle fibre synthesis and nuclear migration during cell division. Additionally, it engages in signal transduction via regulatory/adaptor systems, which are present in several eukaryotic proteins and are involved in the processing of pre-mRNA and the construction of the cytoskeleton[68]. The majority of the treatments (*Aspergillus niger*, *Talaromyces flavus* and *Pseudomonas fluorescens*) had higher expression at 72 hpi, however this was reduced at 96 hpi.

## Conclusion

Biological management of Sheath blight disease has recently emerged as an appealing and promising alternative as use of chemicals are toxic for human health. Present investigation infers that the plant treated with biocontrol agents *viz*. *Chaetomium globosum, Aspergillus niger, Talaromyces flavus*and *Pseudomonas fluorescens* showed higher activity of plant defense related enzymes and also the expression of pathogenicity related genes were found to be less expressed in biocontrol treatments as compared to the inoculated control.

The findings of this study revealed that biological management of *Rhizoctonia solani* caused sheath blight disease in rice *via* induction of the defensive mechanism by plants provides insight into the plant-microbe interaction and a potential technique for plant protection and development in the future.

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## Conflict of interest

All authors declare no conflict of interest.

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