Journal of Biological Control, 40(3): 427-443, 2026, DOI: 10.18311/jbc/2026/55863



Research Article

Gene expression profiling of defence-related enzymes in rice during biocontrol-mediated response against *Rhizoctonia solani* (J. G. Kühn)

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ABSTRACT: Sheath blight, caused by *Rhizoctonia solani*, is a significant fungal disease of rice that leads to considerable yield losses. In the present study, native rhizospheric bacterial isolates from different agro-climatic zones of Telangana were explored for their biocontrol potential against the sheath blight pathogen. A total of twelve isolates, six each of *Pseudomonas* and *Bacillus* were evaluated. *In vitro* confrontation assays indicated that *Pseudomonas fluorescens* strain KMPS1 and *Bacillus subtilis* strain KMBS1 exhibited maximum mycelial inhibition. Molecular identification was confirmed via 16S rRNA and ITS sequencing. Under glasshouse conditions, seed treatment and soil application of the consortial formulation (KMPS1 + KMBS1) significantly improved germination, seedling vigour, and growth parameters. A marked reduction in Per cent Disease Index (PDI) (22.62%) was observed in the consortial treatment, compared to the untreated control (42.11%). Biochemical assays demonstrated elevated levels of defence-related enzymes (PAL, PO, PPO) and total phenolic content in bioagent-treated plants, with maximum activity at 72 h after pathogen inoculation. qRT-PCR analysis revealed strong upregulation of PAL, PPO, and PO genes in consortial treatment, indicating enhanced systemic resistance. The findings highlight the potential of native bacterial bioagents in reducing disease severity and promoting plant growth, offering a sustainable strategy for the biological management of sheath blight in rice.

KEYWORDS: Biocontrol management, host-microbe interaction, rice, rhizosphere bacteria, sheath blight, sustainable management

(Article chronicle: Received: 15-04-2026; Revised:14-08-2026; Accepted: 18-08-2026)

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for nearly 67% of the global population, with Asia accounting for the highest consumption due to dietary preferences (Mohanty, 2013). However, rice productivity is significantly constrained by various biotic and abiotic factors. Among the biotic stresses, fungal diseases pose a critical threat, with *Rhizoctonia solani* Kühn, the causal agent of sheath blight, emerging as a major constraint to sustainable rice production (Webster & Gunnell, 1992).

Sheath blight is considered as the second most damaging fungal disease of rice after blast, capable of causing yield losses ranging from 20 to 50% depending on infection severity (Groth & Bond, 2007; Margani & Widadi, 2018). The pathogen has a wide host range and can survive in soil, plant debris, or alternate hosts by producing sclerotia, making its management particularly challenging (Kozaka, 1965). High nitrogen input, dense planting of high-yielding semi-dwarf varieties and favourable microclimates contribute to its widespread incidence, especially under intensive rice cultivation systems (Savary *et al.*, 1995: Cu *et al.*, 1996).

Traditional management practices for sheath blight include cultural, chemical and biological methods (Yellareddygari *et al.*, 2014). However, chemical fungicides, though widely used, are increasingly viewed as unsustainable due to high costs, resistance development, and environmental concerns. In contrast, biological control using microbial bioagents—particularly bacteria—offers an eco-friendly and cost-effective alternative compatible with integrated disease management strategies.

Bacterial biocontrol agents, notably those from the genera *Bacillus* and *Pseudomonas*, have demonstrated strong antagonistic potential against *R. solani*. These bacteria suppress pathogens via multiple mechanisms. Direct modes include secretion of antimicrobial metabolites, antibiotics, and lytic enzymes, while indirect mechanisms involve the induction of systemic resistance in plants, phytohormone production, and competition for nutrients and ecological niches (He *et al.*, 2021; Kabdwal *et al.*, 2023). Importantly, bacterial agents can provide continuous protection throughout the crop growth period in short-duration crops like rice.

Previous studies have demonstrated the potential of *Pseudomonas fluorescens* and *Bacillus subtilis*, and their combinations for the biological management of rice sheath blight. However, the performance of locally adapted bacterial isolates obtained from rice-growing ecosystems of Telangana, particularly their combined effects on pathogen suppression and host defence responses, remains insufficiently explored. The present study therefore focuses on native *Pseudomonas fluorescens* KMPS1 and *Bacillus subtilis* KMBS1 isolated from rice rhizospheres in Telangana and evaluates their individual and consortial efficacy against *R. solani*. In addition to disease suppression, the study integrates plant-growth promotion, defence-related enzyme activity, and transcriptional responses of PAL, PPO, and PO genes to provide a broader assessment of the potential mechanisms underlying biocontrol efficacy.

Given these advantages, the current research emphasises the use of native bacterial biocontrol agents for the effective biological control of sheath blight disease in rice. The aim is to identify efficient strains capable of suppressing *Rhizoctonia solani*, characterise their mechanisms of action, and evaluate their efficacy under controlled conditions, offering a sustainable approach to disease management in rice ecosystems.

MATERIALS AND METHODS

Rhizospheric soil samples were systematically collected from rice-growing regions across various agro-climatic zones of Telangana, India, taking into account differences in soil type, disease prevalence, and cropping patterns (Figure 1). Both diseased and healthy rice fields were included in the study to ensure a comprehensive representationof the microbial diversity. Within each field, samples were obtained using a random *zig-zag* sampling approach, with three replicates collected per field to ensure accuracy and representativeness, following the methodology

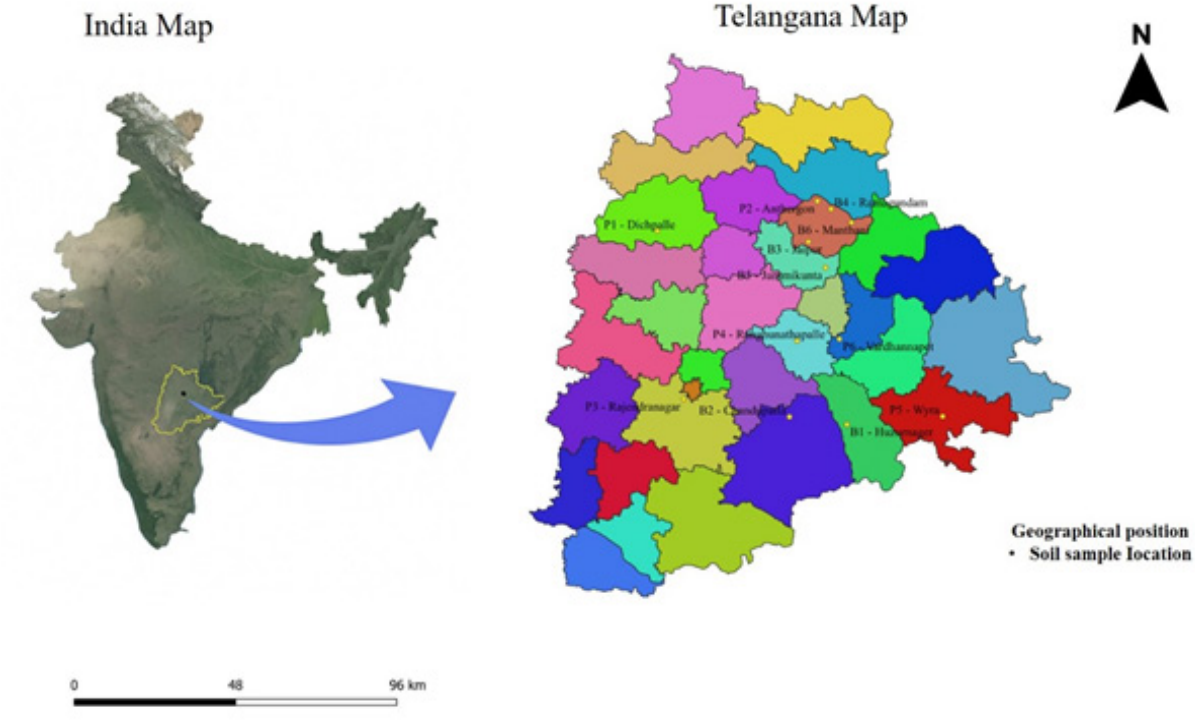


Figure 1. Geographical distribution of rhizosphere soil samples collected from rice fields in Telangana.

of Nawaz *et al.* (2022). The soil closely adhering to the roots (rhizospheric soil) of both healthy and diseased rice plants was carefully excavated. These samples underwent further processing for the isolation of rhizospheric microbes.

Collection, isolation, and purification of native rhizosphere microflora of rice

Microbial cultures obtained from rice rhizospheric soil were maintained using standardised media protocols described by Kannan *et al.* (2022). Fungal isolates were cultured on Potato Dextrose Agar (PDA), while bacterial isolates were sustained on nutrient agar (NA). Additionally, indigenous microbes were isolated from rhizospheric subsoil samples collected across different rice fields using the serial dilution technique (Johnson & Curl, 1977). For this, 1 gram of soil closely associated with rice roots was suspended in 9 mL of sterile distilled water and serially diluted up to 10^{-9}. From the 10^{-6} dilution, 1 mL was plated on nutrient agar (NA) and King's B medium, which served as selective media for isolating *Bacillus* spp. and *Pseudomonas* spp., respectively, following the methodology of Cavaglieri *et al.* (2004). Pure colonies were then sub-cultured and preserved on NA and King's B slants at 4°C for short-term storage, and in 50% glycerol stocks at –80°C for long-term preservation and subsequent experimental use.

Identification of microflora with morphological and biochemical characteristics

Bacterial isolates were classified based on their distinct morphological features and biochemical behaviour, in accordance with the diagnostic framework outlined in *Bergey's Manual of Determinative Bacteriology* (Bergey & Holt, 1994). A suite of standard biochemical assays was employed to further evaluate the bacterial traits. These included tests for ammonia production (Cappuccino & Sherman, 1992), methyl red, indole, catalase, oxidase, citrate utilisation, Potassium Hydroxide (KOH) solubility (Bric *et al.*, 1991), siderophore synthesis (Alexander & Zuberer, 1991), Voges-Proskauer reaction and Hydrogen Cyanide (HCN) production (Lorck, 1948). A summary of the experimental protocols for these tests is provided:

Methyl red test

Following sterilisation, glucose-phosphate broth was inoculated with the respective isolates and incubated at 28 ± 2°C for 48 h, after which five drops of methyl red indicator were added. A red colouration indicated a positive result, whereas yellow signified a negative response.

Indole test

Tryptophan broth was inoculated with overnight bacterial cultures and incubated at 28 ± 2°C for 48 h. After incubation, ten drops of Kovac's reagent were added; the formation of a red layer signified a positive indole test.

Catalase test

A 3% hydrogen peroxide solution was added to a fresh bacterial colony placed on a clean glass slide. Immediate bubble formation confirmed the presence of catalase enzyme activity.

Oxidase test

Bacterial isolates were grown on tryptic soy agar and incubated at 28 ± 2°C for 24 h, and 2–3 drops of Wurster's reagent (tetramethyl-p-phenylenediamine dihydrochloride) were added. A red colour change indicated a positive oxidase result.

Citrate utilisation test

Cultures were streaked on Simmons citrate agar and incubated at 37°C for 48 h. A shift in the colour of the medium from green to blue due to an alkaline pH change was recorded as a positive indication of citrate utilisation.

Voges-Proskauer test

To test tubes containing fully grown cultures, add alpha-naphthol and potassium hydroxide. A cherry-red colour indicated a positive reaction, whereas yellow-brown indicated a negative response.

Hydrogen Cyanide (HCN) production

To evaluate HCN production by *Pseudomonas* spp., isolates were grown in nutrient sucrose medium supplemented with 4.4% glycine, following the method of Lorck (1948). Filter paper soaked in 1% picric acid and 2% sodium carbonate was placed in the lid of the Petri dish and sealed with parafilm. After 48 h of incubation, a yellow to orange colour change in the paper confirmed HCN production.

Siderophore production

The Chrome Azurol S (CAS) assay, following the protocol of Alexander and Zuberer (1991), was employed to evaluate siderophore production. Isolates were incubated on cetrimide agar for 24 h, after which a 0.7% CAS agar overlay was added. Subsequent incubation at 28°C led to a colour change from blue to brownish-orange, indicating siderophore synthesis.

Isolation of pathogens

Sheath blight-infected rice samples were collected from multiple locations across Telangana for the isolation of the causal fungal pathogen. The infected tissues were initially surface sterilised by immersing them in 1% sodium hypochlorite solution for 60 s, followed by three thorough rinses with sterile distilled water to eliminate surface contaminants. Small tissue segments (approximately 0.5 mm), comprising both healthy and infected margins, were then excised, blotted dry on sterile filter paper, and carefully placed onto PDA plates. The inoculated plates were incubated at 28 ± 1°C for 2–3 days, after which emerging fungal mycelia were observed and subcultured to obtain pure isolates. These purified cultures were maintained on PDA slants at 4°C for subsequent studies and long-term preservation.

***In vitro* evaluation of rhizospheric isolates against sheath blight pathogen**

The antagonistic potential of rhizospheric isolates, comprising six strains each of *Bacillus* and *in vitro* antagonism of *Pseudomonas* against the sheath blight pathogen was assessed by streaking a loopful of bacterial culture on both sides of a PDA plate, equidistant from a centrally placed mycelial disc of the pathogen. The plates were incubated in a BOD incubator at 25 ± 2°C for four days, or until the control plate showed full radial growth (up to 9 cm). Post-incubation, pathogen growth was measured to evaluate the extent of inhibition by the bacterial isolates. Following incubation, the radial growth of the pathogen was measured in both treated and untreated plates. The percentage of mycelial inhibition was determined using the formula proposed by Gangwar and Sinha (2010):

$$\text{Mycelia Inhibition (\%)} = \frac{C - T}{T} X\ 100$$

where C represents the radial growth (cm) of the pathogen in the control, and T represents the radial growth in the presence of the bacterial isolate.

Genomic characterisation of bacterial isolates and the fungal pathogen

Among the rhizospheric isolates, *Pseudomonas* strain P2 and *Bacillus* strain B2 demonstrated significant antagonistic activity, each exhibiting 60% inhibition of the sheath blight pathogen in in vitro assays. For molecular identification, the fungal pathogen was grown in Potato Dextrose Broth (PDB), while *Bacillus* and *Pseudomonas* isolates were cultured in LB broth and King's B broth, respectively. From all cultures, genomic DNA was extracted using the NucleoSpin® Genomic DNA Purification Kit (Macherey-Nagel) following the manufacturer's protocol. The quality and concentration of the extracted DNA were evaluated using a NanoDrop spectrophotometer (Thermo

Table 1. List of primers used for amplification of organisms

Organism	Primer	Primer sequence	Primer amplification conditions	No. of cycles	References
Pseudomonas spp.P2	Pseudomonas specific (Ps) primer	Ps-for (5′GGTCTGAGA GGATGATCAGT3′) Ps-rev (5′TTAGCTCCA CCTCGCGGC3′).	Initial denaturation - 5 min, 95°C; Denaturation - 1 min, 94°C; Annealing- 1 min, 57°C; Extension- 1 min, 72°C; Final extension- 10 min, 72°C.	35	Rajwar & Sahgal, 2016
Bacillus spp. B2	16S rRNA universal primer	27F (5'-AGAGTTTGA TCMTGGCTCAG-3') 1492R (5'-CGGTTACCT TGTTACGACTT-3')	Initial denaturation-2 min, 94°C; Denaturation – 40 sec, 94°C; Annealing- 45 sec, 54°C; Extension- 1 min, 72°C; Final extension- 10 min, 72°C.	35	James, 2010
Sheath blight	ITS 1,4	ITS1 (5'TCCTAGGTGA ACCTGCGC3') ITS4 (5'TCCTCCGC TTATTGATATGC3')	Initial denaturation-5 min, 94°C; Denaturation – 1 min, 94°C; Annealing- 1 min, 65°C; Extension- 2 min, 72°C; Final extension- 7 min, 72°C.	30	Ronald*et al.*, 2007

Fisher Scientific) and by electrophoresis on a 0.8% agarose gel pre-stained with ethidium bromide. Subsequently, DNA samples were used for PCR amplification with specific primers listed in Table 1. Each PCR reaction contained 30–50 ng of template DNA, 2.5 mM of dNTP mix, 0.5 µM of each primer, 10 mM Tris–HCl buffer (pH 8.3), 1.5 mM $MgCl_2$, and 5 units of Taq DNA polymerase. Amplification conditions were standardised across all three organisms. PCR products were separated on a 1% agarose gel and compared against a 1000 bp DNA ladder (Novel Gene Technologies Pvt. Ltd.) as a molecular size reference. Visualisation was performed using the BIORAD Molecular Imager Gel DOC™ XR system.

The resulting amplicons were purified using the Promega Wizard® SV Gel and PCR Clean-Up System. Purified DNA samples, with concentrations ranging from 50–100 ng/µl, were then sequenced *via* the Sanger sequencing method at Novel Gene Technologies. The obtained sequences were subjected to BLASTn analysis using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and those showing over 99% identity were classified at the species level. Further computational analyses, including sequence editing, alignment, and phylogenetic inference, were carried out using Clustal Omega (EMBL-EBI) as described by. To assess genetic similarity, sequences grouped within the same dendrogram branch were aligned. Sequences sharing 95–99% identity were considered conspecific, following the guidelines of Marquez *et al.* (2007) and Tamura *et al.* (2011).

In vivo screening of potential antagonists against sheath blight disease under glasshouse conditions

Sterile, pre-soaked seeds of the sheath blight-susceptible rice variety TN1 were treated with bioagent suspensions at a rate of 10 ml/kg seed and incubated for 6 h. Approximately 25 seeds were placed per Petri plate following the standard procedure of the International Seed Testing Association (ISTA). Seeds treated with distilled water served as the negative control. Treated seeds were incubated at 28 ± 2°C for 10 days, following the method described by Vanitha and Umesha (2011). Morphological parameters, including seedling length, seedling dry weight, vigour index-I (germination % × seedling length), and vigour index-II (germination % × seedling dry weight), were recorded at 7, 14, and 21 Days After Sowing (DAS).

For biocontrol studies, 25-day-old seedlings were transplanted into 30 × 25 cm pots containing 5–7 kg of autoclaved soil. At 30 Days After Transplanting (DAT), bacterial and fungal bioagents were applied as a soil drench at a concentration of 2.5 × 10^8 CFU using 10 ml per kg of

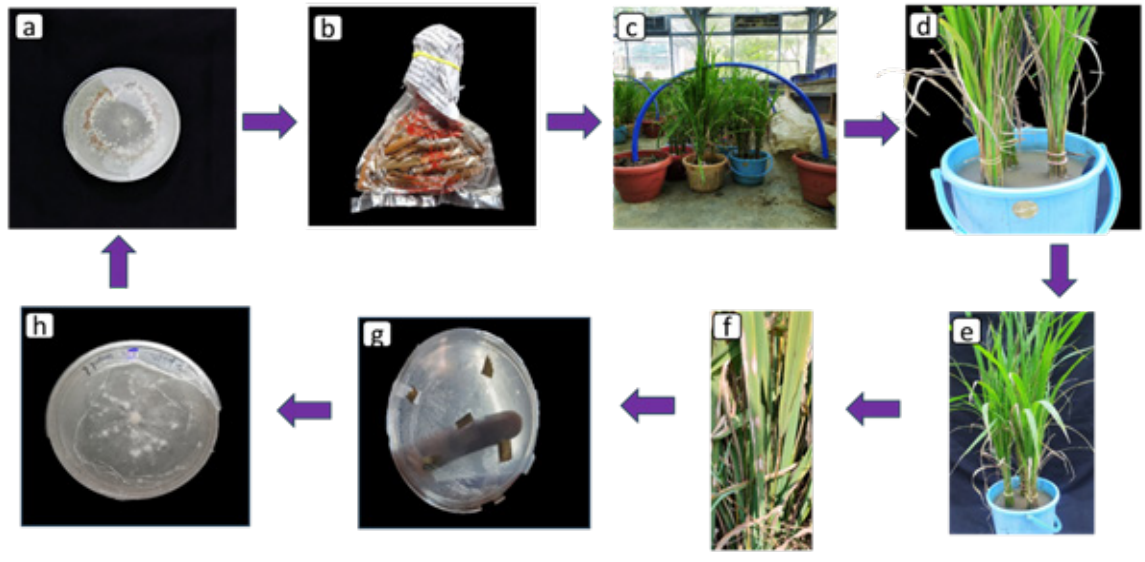


Figure 2. Pathogenicity test (Koch's postulates) of *R.solani* on susceptible rice variety TN1 under glasshouse conditions. **(a).** *Rhizoctonia solani* – pure culture on (PDA); **(b).** Pathogen mass multiplication using typha bits as substrate; **(c).** Glasshouse setup of rice plants for pathogen inoculation; **(d,e).** Inoculated rice plants exhibiting typical sheath blight symptoms; **(f).** Symptoms of sheath blight on infected rice sheath; **(g).** Infected sheath bits for pathogen isolation and **(h).** Pure culture of sheath blight pathogen.

soil. At the maximum tillering stage (45 DAT), *R. solani* was inoculated by placing five 5-mm sclerotial bits colonised by the pathogen per plant (Figure 2), following the protocol of Senapati *et al.* (2022). The experiment was carried out for two consecutive seasons (Kharif & Rabi 2024) with three replications per treatment under controlled glasshouse conditions (85% relative humidity and 30°C). Disease severity was assessed using the PDI, calculated based on the 0–9 Standard Evaluation Scale for sheath blight developed by IRRI (2002).

Sample collection and estimation of defence-related enzymes

Fresh leaf samples were harvested at different time intervals—24, 48, 72, 96, and 120 h after pathogen inoculation (hapi)—from randomly selected plants under each treatment, with three replicates per treatment. Collected samples were placed in sterile polybags, rinsed with sterile distilled water to maintain aseptic conditions, and stored at –80°C until further analysis.

Phenylalanine Ammonia-Lyase (PAL) assay

The activity of Phenylalanine Ammonia-Lyase (PAL) was estimated following the method of Brueske (1980). Fresh leaf tissue (0.5 g) from each replicate was homogenised in 4 ml of 0.2 M borate buffer (pH 8.7) containing 1.4 mM β-mercaptoethanol using a pre-chilled mortar and pestle. The homogenate was centrifuged at 16,000 rpm for 15 min at 4°C. The reaction mixture consisted of 2 ml of 0.2 M borate buffer (pH 8.7), 0.2 ml of 0.1 M L-phenylalanine, and 2 ml of enzyme extract. This mixture was incubated at 32 ± 2°C for 30 min. The reaction was terminated by adding 0.5 ml of 1 M Trichloroacetic Acid (TCA). Absorbance was recorded at 290 nm using a spectrophotometer, and PAL activity was expressed as µmoles of TCA produced per mg fresh leaf tissue per minute.

Peroxidase (PO) assay

Peroxidase (PO) activity was determined as described by Hammerschmidt *et al.* (1982). Fresh leaf tissue (0.5 g) was ground in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0). The reaction mixture consisted of 2 ml of 0.1 M potassium phosphate buffer (pH 7.0), 100 µl of enzyme extract, and 100 µl of 2 mM hydrogen peroxide (H_2O_2). The change in absorbance was monitored at 436 nm at 30-s intervals over a period of 2 min. PO activity was expressed as µmoles per gram of fresh leaf tissue per minute.

Polyphenol Oxidase (PPO) assay

Polyphenol Oxidase (PPO) activity was assessed as per Gauillard *et al.* (1993). Fresh leaves (0.5 g) were homogenised in 5 ml of 0.1 M sodium phosphate buffer (pH 6.5) using a chilled mortar and pestle. The homogenate was centrifuged at 16,000 rpm for 15 min at 4°C, and the supernatant was used as the enzyme extract. The assay mixture contained 0.4 ml of enzyme extract, 0.4 ml of 0.01 M catechol (substrate), and 3.0 ml of 0.1 M sodium phosphate buffer (pH 6.5). After incubation at 28 ± 2°C for 5 min, the absorbance was measured at 495 nm every 30 s for 3 min. PPO activity was expressed as µmoles per gram of fresh leaf tissue per minute.

Total Phenol Content (TPC) assay

Total phenol content was quantified using a modified method of Malick and Singh (1980). Leaf tissue (0.1 g) was homogenised in 5 ml of 50% methanol and incubated for 1 h. The homogenate was centrifuged at 13,000 rpm for 15 min. From the resulting methanolic extract, 0.1 ml was diluted to 1 ml with distilled water, followed by the addition of 0.5 ml of Folin–Ciocalteu reagent. After 15 min, 1 ml of 20% sodium carbonate solution was added, mixed thoroughly, and incubated at room temperature for 1 h. Absorbance was measured at 725 nm, and total phenol content was expressed as µg Gallic Acid Equivalents (GAE) per gram of fresh weight.

Quantitative real-time PCR (qRT-PCR) analysis of defence gene expression

Total RNA was extracted from rice leaf samples of nine treatments (T1–T9) at 0, 24, 48, and 72 h after inoculation (hai) with *R. solani*, including non-inoculated controls. Samples were flash-frozen in liquid nitrogen and stored at –80°C. RNA was isolated using Trizol reagent (Takara, Japan), treated with DNase I, and quantified using a NanoDrop 2000. First-strand cDNA was synthesised using the HiScript® 1st Strand cDNA Synthesis Kit (Vazyme, China). qRT-PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems) using gene-specific primers (Table 2), 2 µL diluted cDNA, 10 µL SYBR Green I Master Mix (Takara), and 7.2 µL ddH_2O per reaction. Each sample had three biological and three technical replicates. Actin was used as the internal control (Forward Primer (Actin-q-F): 5'-CTTCATAGGAATGGAAGCTGCGGGTA-3', Reverse Primer (Actin-q-R): 5'-CGACCACCTTGATC TTCATGCTGCTA-3'). Relative gene expression was calculated using the 2^–ΔΔCt method, assuming consistent

Table 2. Primers used for defense gene expression analysis

PAL	**PAL F**-5'-GGTGTTCTGCGAGGTGATGA-3' **PAL R**-5'-AGGGTGCTTCAGCTTGT-3'	Vichekaet *al.*, 2017
PO	**PO F**- 5′- GCTCTTCCACGATGTTGAGG -3′ **PO R**-5′- TGACCGTTGTCGTACTTGGA -3′	Xiaowanet *al.*, 2022
PPO	**PPO-F-** 5′- TGACGAAGTGTGATGGCAAC -3′ **PPO-R-** 5′- CCTCTGCGTTGTTGATGTTG -3′	Xiaowanet *al.*, 2022

amplification efficiency (95%) and stable reference gene expression (This experiment relied on a housekeeping gene (Actin)). Fold change (FC) values >1 indicate upregulation, <1 indicate downregulation, and 1 indicates no change.

STATISTICAL ANALYSIS

The experiments were laid out in a Completely Randomised Design (CRD), with three replications for each treatment, and the data were analysed using one-way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at a 5% significance level ($P \leq 0.05$) using OPSTAT (Dafaallah, 2019). R software was used for generating heatmaps and boxplots (Makowski *et al.*, 2020), while QGIS was employed for mapping the geographical coordinates of the rhizosphere sampling sites. For the glasshouse disease experiment, each treatment consisted of three independent pots/replications, with 3 plants per pot;for seedling growth assessment, data were obtained from 10 seedlings per treatment.

RESULTS

Isolation and identification of microflora with morphological and biochemical characteristics

Rhizospheric microflora isolated from different agro-climatic zones of Telangana (Figure 1, Table 3) differ in their morphological characters like colony shape, size and

Table 3. Cultural and Morphological characteristics of *Bacillus,Pseudomonas* grown on Nutrient Agar (NA), King's B media base (KB) medium after 72 h of incubation

S. No.	Isolates	Time of growth (h)	Shape of the colony	Colony colour	Colony elevation	Surface of the colony	Margin of the colony	Gram reaction	Shape of the isolate	Spor-ulation
1	P1	19±0.51^{x}	Round	Yellowish green	Convex	Smooth	irregular	Negative	Rod	Negative
2	P2	35±0.39^{x}	Irregular	White	Convex	Smooth and shiny	irregular	Negative	Rod	Negative
3	P3	23±0.59^{x}	Irregular	Dull white	Convex	Smooth	irregular	Negative	Rod	Negative
4	P4	20± 0.51^{x}	Round	Yellowish green	Convex	Smooth and shiny	irregular	Negative	Rod	Negative
5	P5	22±0.58^{x}	Round	white	Raised	Smooth and mucoid	irregular	Negative	Rod	Negative
6	P6	31±0.66^{x}	Round	Yellowish green	Convex	Smooth and shiny	irregular	Negative	Rod	Negative
7	B1	20±0.51^{x}	Oval	Dull grey	Oval	Opaque and dry	irregular	positive	oval	positive

Table 3. Continued

8	B2	36±0.38[x]	Ellipsoidal	Off white	Convex	Smooth and moist	irregular	positive	Elli-psoidal	Negative
9	B3	30±0.64[x]	Rod	White	Flat	Smooth and shiny	irregular	positive	Rod	Negative
10	B4	18 ± 0.50 e	Rod	Off white	Convex	Smooth	irregular	positive	Rod	Negative
11	B5	21 ± 0.57 d	Oval	White	Flat	Opaque	irregular	positive	Oval	Negative
12	B6	24 ± 0.58 c	Rod	White	Oval	Smooth and dry	irregular	positive	Rod	Negative

* Meanoffivereplications;numericalvalueswithdifferentlettersaresignificantlydifferent(p<0.05,DMRT,OPSTAT)

Table 4. Biochemical characterisation of collected bacterial isolates

S. No.	Isolates	Indole	MR	VP	Citrate utili-sation	Catalase	oxidase	Starch hydrolysis	Gelatin lique-faction	HCN	Side-rophore prod-uction
1	P1	+	-	-	+	+	+	-	+	-	+
2	P2	+	-	-	+	+	+	-	+	+	+
3	P3	-	-	-	+	+	+	-	+	+	+
4	P4	+	-	-	-	+	+	-	+	+	+
5	P5	-	-	-	+	+	+	-	+	-	+
6	P6	+	-	-	-	+	+	-	+	-	+
7	B1	-	-	+	+	+	+	+	+	-	-
8	B2	-	-	+	+	+	+	+	+	+	+
9	B3	-	-	+	+	+	+	+	+	-	+
10	B4	-	--	+	+	+	+	+	+	-	+
11	B5	-	-	+	+	+	+	+	+	-	-
12	B6	-	-	+	+	+	+	+	+	+	-

+= Positive,-=Negative, MR=MethylRed,VP=VogesProskauer,HCN=Hydrogen Cyanide

pigmentation. The isolates were similar to the potential bioagent *Pseudomonas* spp. (6) and *Bacillus* spp. (6). Biochemical studies revealed the presence of enzymes catalase, oxidase, etc., indicating the antagonistic potential of the microbes studied (Table 4).

In vitro antagonism of sheath blight by potential bioagents

Under *in vitro* conditions, antagonistic activity of twelve native rhizospheric bacterial isolates (six *Pseudomonas* spp.

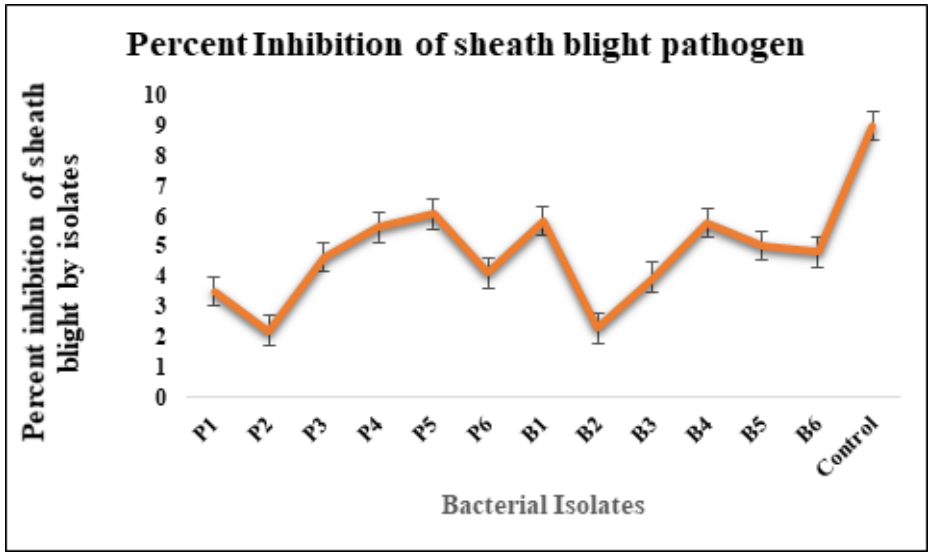


Figure 3. Preliminary screening of collected bacterial isolates against sheath blight pathogen.

and six *Bacillus* spp.) against *R. solani*, the sheath blight pathogen, was evaluated using the dual culture technique. The radial growth of the pathogen in the presence of the test isolates ranged from 2.20 cm to 6.07 cm, compared to 9.00 cm in the control. Among the *Pseudomonas* isolates, P2 recorded the highest inhibition of mycelial growth with 75.55% (2.20 cm), followed by P1 (61.00%, 3.51 cm), P6 (54.33%, 4.11 cm), P3 (48.33%, 4.65 cm), P4 (37.55%, 5.62 cm), and P5 (32.55%, 6.07 cm). Among the *Bacillus* isolates, B2 showed the maximum inhibition with 74.66% (2.28 cm), followed by B3 (55.88%, 3.97 cm), B6 (46.55%, 4.81 cm), B5 (44.22%, 5.02 cm), B4 (35.88%, 5.77 cm), and B1 (35.22%, 5.83 cm). All the isolates exhibited significant inhibition of the pathogen compared to the control, indicating their potential antagonistic nature against *R. solani* (Figure 3).

Molecular identification of bacterial isolate and fungal pathogens

The 16S rDNA sequences of two bacterial isolates and the ITS region of the sheath blight pathogen were analysed through NCBI BLAST. Isolate P2 showed 100% similarity with *Pseudomonas fluorescens*, while B2 matched 100% with *Bacillus subtilis*. These isolates were hence designated as *P. fluorescens* KMPS1 (NCBI Accession No: PV687531) and *B. subtilis* KMBS1 (NCBI Accession No: PV687527) for future reference. Phylogenetic analysis revealed that P2 shared 98.5% sequence homology with closely related

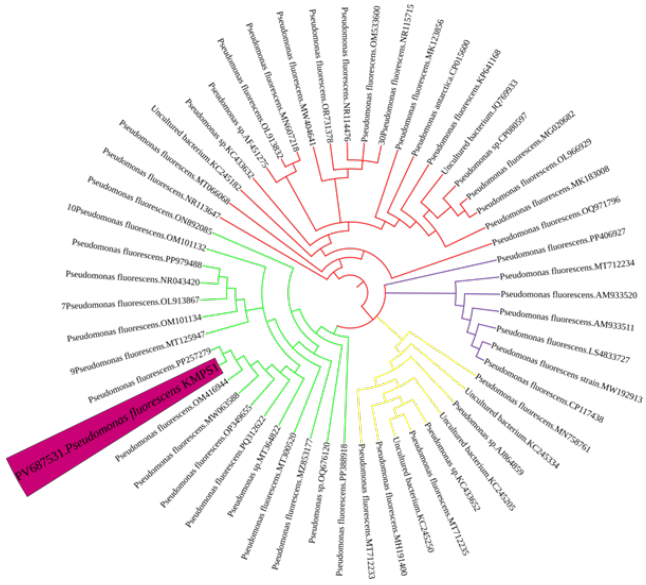


Figure 4. Phylogenetic tree of 16S rRNA gene sequences for *Pseudomonas fluorescens* strain KMPS1 constructed with related isolates from NCBI database.

Pseudomonas species (Figure 4), whereas B2 exhibited 100% sequence similarity with its closest *Bacillus* relative (Figure 5). The ITS region of the sheath blight pathogen showed 99.56% similarity with *R. solani*, confirming its identity, and was designated as *R. solani* KMPS1 (NCBI Accession No: PV687533) (Figure 6).

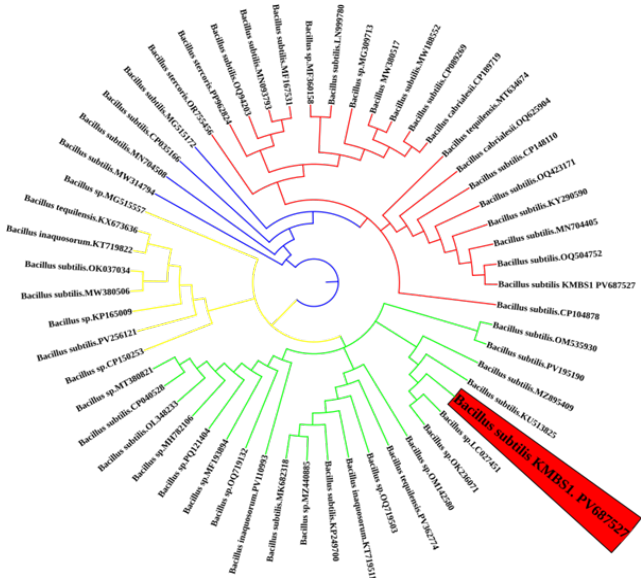


Figure 5. Phylogenetic tree of 16S rRNA gene sequences for *Bacillus subtilis* strain KMBS1 constructed with related isolates from NCBI database.

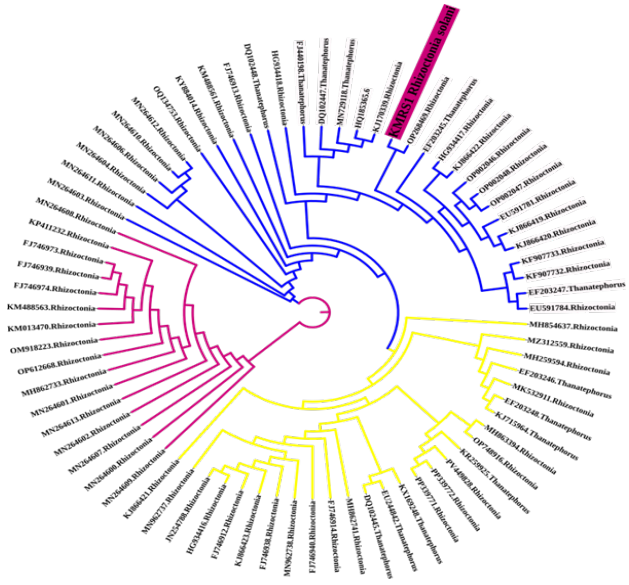


Figure 6. Phylogenetic tree of ITS gene sequences for *Rhizoctonia solani* strain KMRS1 constructed with related isolates from NCBI database.

In vivo screening of potential antagonists against sheath blight disease under glasshouse conditions

Plants treated with the consortia of two isolates were found to have 100% seed germination with seedling vigour index-1 (3812) and vigour index-2 (18.10), and more dry weight (0.191g) when compared to the control shows 80% germination with VI-1 1658.6, VI-23.16 and 0.061g dry weight. The consortia of both isolates were found to induce maximum growth promotion, followed by KMBS 1 and KMPS 1 (Table 5).

In vivo experiments conducted under glasshouse conditions demonstrated a significant reduction in sheath blight severity with the application of native bioagents. Among the treatments, the lowest PDI was observed in the consortial treatment with 22.62%, followed by KMPS1 alone with 20.32%. The control treatment with only the pathogen showed a PDI of 68.45%, indicating severe disease incidence in the absence of any treatment. These results clearly highlight the biocontrol potential of native isolates, especially KMPS1 and its consortia with KMBS1,

Table 5. Screening of plant growth promotion activities using different bacterial bioagents in TN1 variety

Isolates	Root length (cm)	Shoot length (cm)	Germi-nation (%)	Seedling length (cm)	Vigour index- I	Vigour index- II	Dry weight(g)
KMBS 1	12.07^{b}	16.11^{c}	98^{b}	28.18^{c}	2798.88^{c}	13.32^{c}	0.136^{a}
KMPS 1	14.07^{c}	18.13^{b}	100^{a}	32.20^{b}	3262.01^{b}	16.10^{b}	0.161^{a}
Consortia	17.26^{a}	21.31^{a}	100^{a}	39.24^{a}	3972.01^{a}	19.20^{a}	0.192^{a}
Control	8.11^{d}	12.17^{d}	80^{c}	20.29^{d}	1669.61^{d}	5.76^{d}	0.072^{b}
CD	0.037	0.037	1.15	0.08	1.22	0.069	0.002
CV	0.2	0.1	1.5	0.3	0.03	0.08	0.67

Data represent the mean ±SE of 10 seedlings. Numerical values with different letters are significantly different ($p< 0.05$, DMRT, OPSTAT)

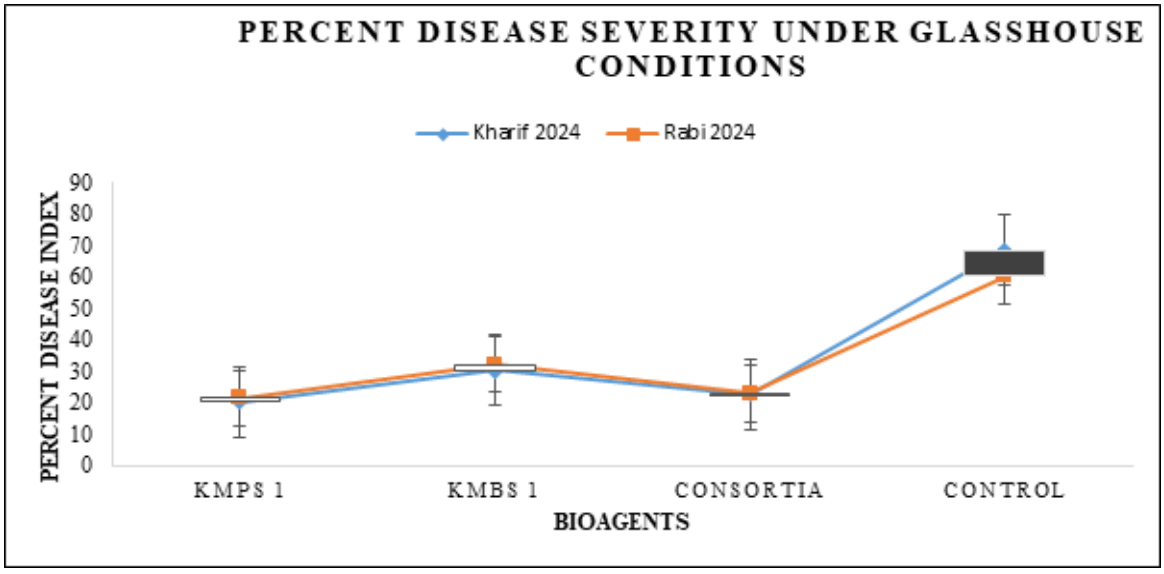


Figure 7. Effect of the potential bioagents on sheath blight disease severity under glasshouse conditions.

in managing sheath blight under controlled conditions. (Figure 7).

Estimation of biochemical parameters

Plants treated with bioagents were studied for defenceenzyme activity, and also estimated its quantity at different time intervals. The defenceenzyme activity was found to increase after 24 h of the given treatment, attained maximum activity at 72 h and started declining from then. However, the highest activity of PAL, PO, PPO, and total phenol was seen in the treatment T6- seed treatment with consortia of KMPS1, KMBS1+ Soil application of consortia + *R.solani,* followed by treatment T3-Seed treatment with consortia of KMPS1 and KMBS1 (Figure 8).Each plot is descriptive of 1 treatment. The highest PAL was recorded in plants treated with consortia of bioagents and challenge-inoculated with the sheath blight pathogen (T6), which varied at 24 h, 74 h and 120 h (3.070, 3.761, 3.450 µg cinnamic acid/g leaves/min, respectively). Minimum activity was recorded in the control plant alone (T8) varies at 24 h, 74 h and 120 h (1.350, 1.761, 1.470 µg cinnamic acid/g leaves/min, respectively). Peroxidase activity was recorded highest in the treatment (T6) which varies at 24 h, 74 h and 120 h (0.650, 0.690, 0.671 Δ Absorbance 470nm/ g leaves/min, respectively), lowest activity of peroxidase at all intervals *viz.*, 24 h, 72 h and 120 h (0.04, 0.07 and 0.06 Δ Absorbance 470nm/g leaves/min, respectively) was found in treatment T8 (Control). Polyphenol oxidase was recorded to be highest in the treatment (T6) varies at 24 h, 74 h and 120 h (0.051, 0.073 and 0.062 Δ Absorbance 280nm/ g leaves/ min, respectively), lowest activity of peroxidase at all intervals *viz.*, 24 h, 72 h and 120 h (0.001, 0.025 and 0.017 Δ Absorbance 280nm/ g leaves/min, respectively) was found in treatment T8 (Control). Maximum activity of total phenol content was observed in (T6) varies at 24 h, 74 h and 120 h (2.572, 4.532 and 3.611 Δ Absorbance 650nm/µg phenols/g leaves/min, respectively), lowest activity of peroxidase was recorded in T8 (Control) at all intervals *viz.*, 24 h, 72 h and 120 h (0.583, 1.581 and 1.080 Δ Absorbance 650nm/µg phenols/g leaves/min, respectively).

Transcription activity of defence genes upon bioagent application

The expression profiles of three key defence-related genes—Polyphenol Oxidase (PPO), Phenylalanine Ammonia-Lyase (PAL), and Peroxidase (PO)—were analysed in rice plants challenged with *R. solani* at four time points: 0, 24, 48, and 72 h after inoculation (hai), under nine different treatments (T1–T9). Heatmaps were generated to visualise relative gene expression levels (Figure 9). The colour gradient in the heatmaps represents normalised expression values: positive values (*e.g.*, +1 to +1.5) indicate upregulation (depicted in warm colours such as yellow, orange, and purple), while negative values (*e.g.*, -1) represent downregulation (shown in cooler colours like blue, green, or red), and a value of zero indicates baseline expression.

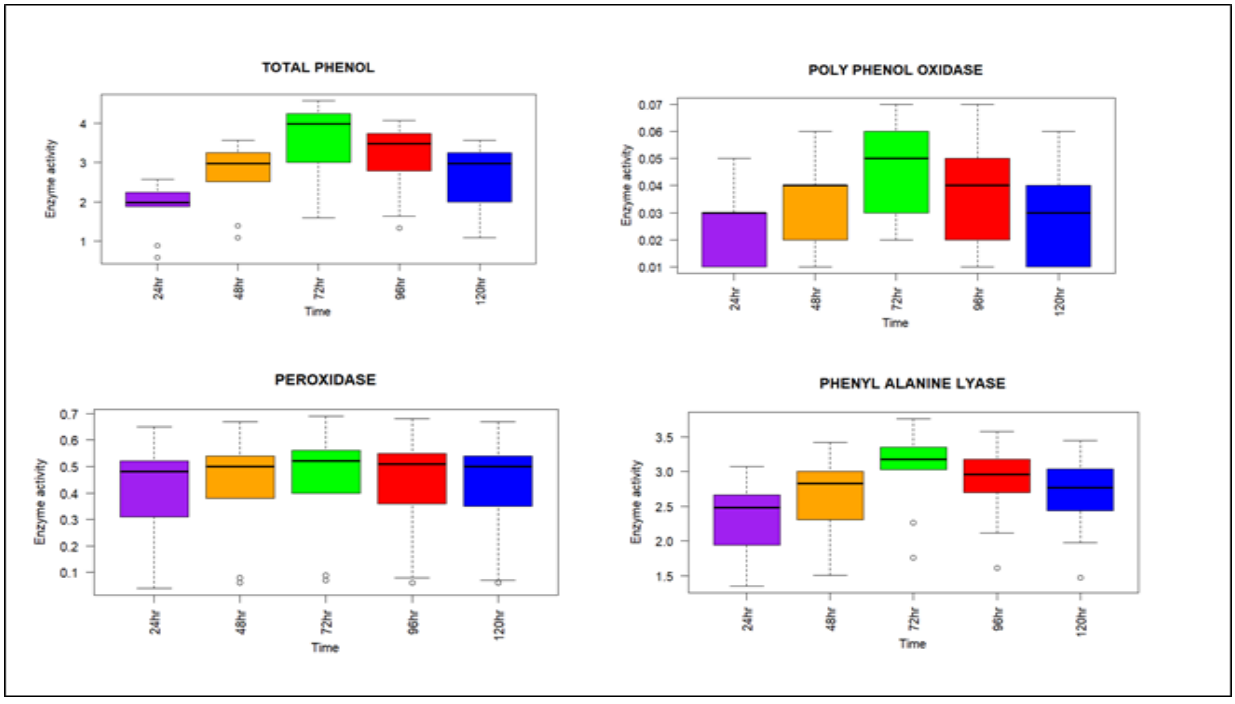


Figure 8. Box plot illustrate the changes in defense enzymes upon challenge inoculation with *R. solani.*

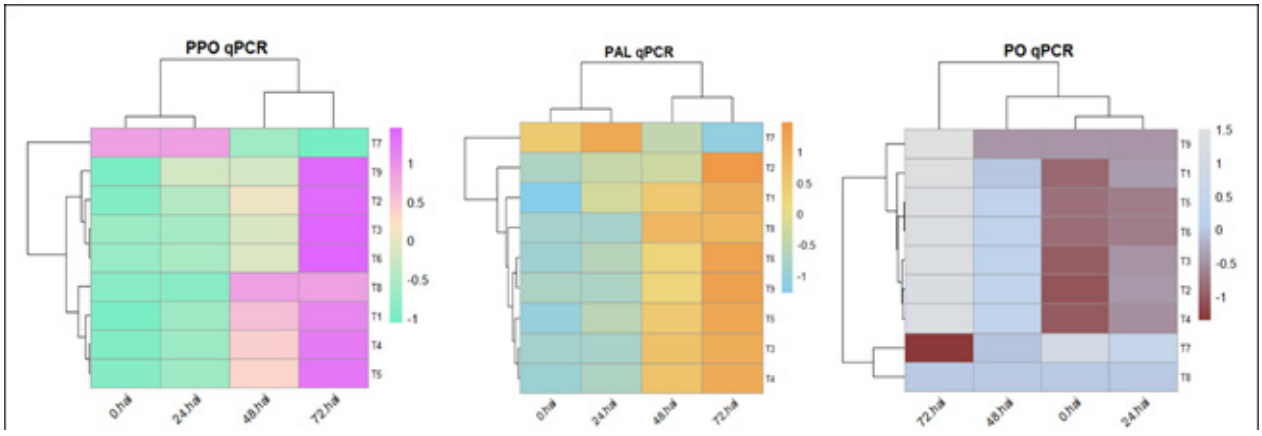


Figure 9. Heatmap representation of qRT-PCR-based expression profiles of defense-related genes (PPO, PAL, PO) in rice across different treatments and time points.

Note: Heatmaps depict the relative expression levels of Polyphenol Oxidase (PPO), Phenylalanine Ammonia-Lyase (PAL), and Peroxidase (PO) genes in rice plants subjected to different treatments (T1–T9) at 0, 24, 48, and 72 h after inoculation (hai) with *Rhizoctonia solani*. Expression data were normalised and visualised using hierarchical clustering.

The colour scale represents log2 fold-change values:

- +1 to +1.5 (warm colours such as orange, purple, red) indicate upregulation of gene expression,
- -1 (cool colours like blue, green, teal) indicate downregulation, and
- 0 (white or neutral shades) denotes baseline or no significant change in expression compared to control.

Among the treatments, T6 (seed treatment with consortia + soil application of consortia + *R. solani*) consistently exhibited the highest expression for all three genes: PAL (2.5-fold at 0 hai to 48.5-fold at 72 hai), PPO (1.3-fold at 0 hai to 33.5-fold at 72 hai), and PO (2-fold at 0 hai to 10.2-fold at 72 hai). Treatments T4 and T5, which involved individual bioagents combined with *R. solani*, also showed substantial upregulation: T4: PAL (1.8–23.3 fold), PPO (1.5–14.6 fold), PO (1–7.1 fold); T5: PAL (1.5–17.7 fold), PPO (1–8.6 fold), PO (2–6.2 fold). Moderate expression levels were observed in the seed-only treatments (T1–T3), indicating partial induction. The negative control (T7) showed consistently low expression across all genes and time points, confirming the absence of defence activation. Meanwhile, the healthy (T8) and fungicide-treated (T9) controls exhibited minimal to basal expression levels. These results clearly demonstrate that the application of biocontrol agents—especially in consortia form—significantly activates host defence pathways, indicating the induction of systemic resistance in rice plants against *R. solani*.

DISCUSSION

A significant constraint to sustainable rice production in India is the widespread occurrence of fungal diseases. These pathogens frequently cause severe epidemics in major rice-growing regions, resulting in yield losses exceeding 60% (Nayak *et al.*, 2017). The adoption of biocontrol strategies using bacterial bioagents has emerged as a promising and eco-friendly alternative to chemical methods. These approaches involve the application of disease-suppressive bacteria that not only inhibit pathogen activity but also enhance plant growth. In our study, greenhouse experiments demonstrated the efficacy of bacterial bioagents in promoting rice growth and suppressing sheath blight disease. Notably, Pseudomonas isolates were observed to produce important plant growth regulators such as cytokinins, gibberellins, and Indole Acetic Acid (IAA), which can directly or indirectly influence plant growth and development (Barbaccia *et al.*, 2022).

The isolated *Pseudomonas* and *Bacillus* strains exhibited morphological diversity, including colony pigmentation (pale yellow or white), shape (convex or flat), and margin characteristics (smooth or irregular) when cultured on King's B and Nutrient Agar (NA) media. These differences may be attributed to the varying agro-climatic conditions—such as temperature, humidity, and soil type—of the regions from which the strains were isolated (Soesanto *et al.* 2011). Physical and chemical properties of the soil, including cropping patterns and pH levels, are known to influence microbial diversity and community structure (Furtak *et al.* 2020).

The isolates also varied in their biochemical characteristics, which were determined using standard biochemical and enzymatic assays, including tests for Voges-Proskauer (VP), catalase, and oxidase activity. These are commonly employed as confirmatory tests to differentiate between Gram-positive and Gram-negative bacteria. Additionally, Hydrogen Cyanide (HCN) production was assessed, as it is a key trait of microbial bioagents with antimicrobial properties (Flores-Treviño *et al.* 2019). Enzymes such as catalase and oxidase play a role in detoxifying reactive oxygen species like hydrogen peroxide, thereby contributing to microbial survival and antagonistic potential. HCN acts directly against pathogens by inhibiting metal-containing enzymes like cytochrome c oxidase involved in the respiratory chain (Flores-Treviño *et al.* 2019).

Among the bacterial strains tested, nine isolates produced statistically significant quantities of siderophores—low molecular weight, high-affinity iron-chelating compounds. These siderophores enhance iron uptake by solubilising ferric ions (Fe^{3+}), thereby making iron more accessible to the host plant while limiting its availability to pathogens. Since iron is essential for pathogen growth, this mechanism provides a dual benefit: enhanced plant nutrition and reduced pathogen virulence (Parveen & Latha 2019).

For molecular characterisation, fungal strains were identified using Internal Transcribed Spacer (ITS) region sequencing, a widely accepted approach for differentiating fungal genera. For bacterial strains, 16S rRNA gene sequencing was employed, allowing for accurate species-level identification and phylogenetic analysis (Kumar *et al.* 2015; Sun *et al.* 2008).

Seed treatment with bacterial bioagents significantly improved seed germination and seedling vigour under in vitro conditions compared to untreated controls. Among the tested strains, *Pseudomonas fluorescens* exhibited the highest germination percentage and vigour index. These strains consistently reduced disease incidence over two experimental seasons (Kharif and Rabi 2024) under greenhouse conditions. In the present study, novel bacterial strains were isolated and characterised from infected rice fields, and these strains demonstrated the ability to inhibit

the growth of *R. solani*, the causal agent of sheath blight in rice.

The selected strains KMPS1 (*Pseudomonas*) and KMBS1 (*Bacillus*) demonstrated strong antagonistic activity against *R. solani* both in vitro and in vivo. This could be attributed to their ability to secrete antimicrobial compounds such as bacteriocins, phenazines, and HCN. These metabolites are known to inhibit the growth of pathogenic fungi (Caulier *et al.* 2019; Adrien & Martin 2018). Furthermore, competition for nutrients and secretion of lytic enzymes like chitinases, proteases, pectinases, and glucanases—known to degrade pathogen cell walls—also contributed to their antagonistic efficacy (Pandit *et al.* 2022).

Suppression of *R. solani* was observed, leading to a marked reduction in percent disease index compared to the untreated control. Application of KMPS1 and KMBS1 resulted in the complete death of mycelia within 48 h. The sclerotial bodies failed to germinate upon the application of bioagents. sclerotial bodies—compact masses of hyphae that serve as persistent survival structures. These sclerotia enable the pathogen to survive in soil and plant debris for several years, even in the absence of a host, making it a primary source of inoculum in subsequent cropping seasons (Jiehua *et al.* 2019; Lu *et al.* 1994; Wang *et al.* 2019). The ability of the biocontrol agent to effectively disrupt these sclerotial structures underscores its potential as a sustainable disease management strategy for sheath blight.

The enhanced efficacy observed in the consortial application of *P. fluorescens* KMPS1 and *B. subtilis* KMBS1 may be attributed to their complementary modes of action. While *Pseudomonas* spp. are known for the production of antibiotics, siderophores, and phytohormones, *Bacillus* spp. contribute through robust enzyme secretion and spore-forming ability, ensuring prolonged rhizosphere colonisation. The combination likely resulted in multi-target pathogen suppression and a stronger induction of systemic resistance.

The elevated activity of PAL, PO, and PPO enzymes in treated plants was strongly correlated with the transcriptional upregulation of corresponding genes, as confirmed by qRT-PCR. Notably, consortial treatment (T6) led to the highest gene expression levels at 72 h after inoculation, aligning with peak enzymatic activity. This suggests a direct role of KMPS1 and KMBS1 in enhancing systemic resistance by triggering defence-related gene expression pathways.

Plant defence enzymes were also found to play a pivotal role in disease resistance. Phenylalanine ammonia-lyase (PAL), the first enzyme activated in the phenylpropanoid pathway, serves as a precursor to several secondary metabolites, including phytoalexins, lignins, and flavonoids. Peroxidase (PO) and polyphenol oxidase (PPO) are involved in the oxidative polymerisation of phenolic compounds and cross-linking of cell wall proteins, thereby strengthening the plant's physical barriers against pathogen invasion. Furthermore, total phenolic content, comprising a range of antimicrobial compounds such as tannins and flavonoids, was elevated in treated plants, indicating an enhanced systemic defence response (Rais *et al.* 2017; Wiesel *et al.* 2014).

CONCLUSION

Native rhizospheric isolates KMPS1 (*Pseudomonas fluorescens*) and KMBS1 (*Bacillus subtilis*) demonstrated strong antagonistic activity against *Rhizoctonia solani*, the causal agent of sheath blight in rice. Their consortial application not only enhanced disease suppression but also significantly improved plant growth and defence responses. Furthermore, qRT-PCR–based transcriptomic analysis revealed the upregulation of key defence-related genes, providing molecular evidence for the induced resistance and overall effectiveness of the combined treatment.

ACKNOWLEDGEMENT

We acknowledge the support received from the Management and Principal, Vaagdevi Degree & PG College (Autonomous) to carry out the research work.

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AUTHOR CONTRIBUTION

The conception and design of the study, data acquisition, analysis, and interpretation were carried out by Koteshwar Pagilla. The manuscript was drafted and critically revised for important intellectual content by Sateesh Suthari. All authors have read and agreed to the published version of the manuscript.