Diversity analysis of antagonistic microbes against bacterial leaf and fungal sheath blight diseases of rice

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Abstract

Background: Biocontrol is an effective strategy in the integrated management of plant diseases, now more as a necessity than choice, in the present era of environmental and health awareness. Microbial diversity is a wonder by nature that inspires to explore and accordingly, the diversity analysis of the isolated microbes revealed their morphological and molecular differences. The DNA provides a common platform to store the microbial information in the form of databases in public domain that can be used by anyone from anywhere.

Results: Exploration for native microbes in the present study resulted in isolation of different isolates of Trichoderma and Bacillus. The microbes were identified using morphological traits and molecular markers and the key conserved 18S and 16S gene sequences submitted with the appropriate repositories. Nucleotide analysis indicated a close phylogenetic relationship between BIK 2 and BIK 3 (Bacillus isolates) and within all the 5 Trichoderma isolates. The percent disease reduction of Rhizoctonia solani and Xanthomonas oryzae pv. oryzae (Xoo) was more in plants treated with consortia of the Trichoderma (61.13%) and Bacillus (53.59%) isolates, respectively. Screening of plant growth promotion activities, percentage increase in root (41.00%) and shoot length (44.77%) were found to be maximum in Trichoderma consortia treated plants.

Conclusions: Three Bacillus and one Trichoderma strains, viz., B. velezensis, B. subtilis and B. paralicheniformis and Trichoderma asperellum, were identified and found to be effective against R. solani and Xoo pathogens of rice. In vitro and in vivo studies indicated that TAIK1 and BIK3 were found to be the most potential isolates among others isolated. Ability to improve plant growth was more pronounced by consortia of microbes.

Keywords: Biocontrol, Trichoderma, Bacillus, Diversity analysis, Bacterial blight, Sheath blight

Background

Agricultural soils are the most dynamic in nature; however, the micro-flora and fauna that impacts the quality and yield of the crops grown generally remains a mystery. Increasing population and decreasing agricultural soil availability result in an undue increase in demand for food, inviting an extremely intense cultivation. This led to the use of more chemical inputs causing severe stress on the environment and human health (Chukwu et al. 2019). In this context, the use of microbes as biopesticides to protect them from diseases, improve yield quality in a sustained eco-friendly manner, plays a very important role in the process of providing food security for the ever-increasing population (Mukherjee et al. 2013).

Rice is the leading staple crop of the world and consumed by more than half of Indian population. It is attacked by various fungal, bacterial and viral pathogens, incurring huge loss to crop quality and quantity (Kölh et al. 2019). Development of resistant varieties,
wherever possible, is considered a welcome replacement for synthetic pesticides. However, there are some diseases like sheath blight and false smut in rice, for which donors with strong resistance are not available. In addition, the breakdown of resistance in diseases like bacterial blight of rice (BB) and blast leads to significant loss to the farmers (Chukwu et al. 2019). Biological control using friendly microbes or their products to suppress the pathogens plays a crucial role in sustainable integrated management of plant diseases (Gnanamanickam 2009). Species belonging to the genera *Trichoderma*, *Bacillus* and *Pseudomonas* are more commonly found in the plant rhizosphere that help in the growth promotion of the plants and induce resistance/tolerance against biotic and abiotic stresses. These microbes suppress the pathogens either directly by contact or indirectly by releasing certain chemical compounds and releasing plant growth promoting hormones that helps in healthy growth and development of crops (Abo-Elyousr et al. 2019). They elicit defence system in plants by activating signal molecules that typically recognize pathogen, stimulate and initiate defence pathways. Use of conserved sequences, viz., 16S rRNA for bacterial isolates and internal transcribed spacer (ITS) regions for fungal isolates, complements their phenotypic characterization (Ashe et al. 2014). In addition, phylogenetic studies based on taxonomic markers specific for individual species and their multiple genes have been described as useful tools for molecular diversity studies (Youssuf et al. 2014). In the present study, specific primers for detection of endonuclease genes have been deployed in addition to primers for 16S rRNA to identify different isolates of *Bacillus*. Similarly, 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene sequences were deployed for identification of *Trichoderma* at species level. The present study was to isolate native *Trichoderma* and *Bacillus* isolates from the rhizosphere of different rice growing regions of Telangana, India, establishing their identity, potential for growth promotion and efficiency to suppress *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Rhizoctonia solani*, the 2 major pathogens causing bacterial blight and sheath blight diseases of rice, respectively.

**Methods**

**Microbial preparation**

*Trichoderma* and *Bacillus* isolates were obtained from the rice rhizosphere of different regions of Telangana State, using standard serial dilution method. *Trichoderma* specific medium (TSM) and peptone yeast extract medium (PYEM) were used as selective medium for isolation and purification of fungal and bacterial antagonists, respectively (Cavaglieri et al. 2004). Key morphological and microscopic characters were used for identification of *Trichoderma* isolates (Gams and Bissett 1998), and *Bacillus* isolates (Sneath 1986). Scanning electron microscopy (SEM) was done as described by Bozolla and Russell (1999). Samples were fixed in 2.5% glutaraldehyde for 24 h at 4 °C, followed by 2% aqueous osmium tetroxide for 4 h. After dehydration in series of graded alcohols, the samples were mounted and observed in SEM (JEOL JSM-5600).

**Pathogens**

*R. solani*, collected from Plant Pathology Laboratory, Hyderabad (Yugander et al. 2015) and *Xoo* (Accession number: MZ158566), were used for the experimental study. Their pathogenicity was proved according to Koch’s postulates on TN1 cultivar.

**Genomic DNA from bacterial and fungal antagonists**

*Bacillus* isolates, viz., B2, B3 and B4, and *Trichoderma* isolate T7 were cultured in Luria Bertani (LB) and potato dextrose broth (PDB), respectively. *Bacillus* cells were harvested by centrifuging at 8000 × g for 10–15 min and fresh mycelial mats of *Trichoderma* were used for the isolation and purification of genomic DNA. NucleoSpin® genomic DNA isolation and purification kit (Machery-Nagel) were used as per the manufacturer’s instructions. The isolated DNA was quantified using both nanodrop spectrophotometer (Thermofischer) and 0.8% agarose gel pre-stained with ethidium bromide.

**PCR amplification**

*Bacillus* isolates were identified using amplification of 16S rRNA (~1500 bp) by universal primer pairs 27F (5′-AGAGTTTGATCMTGCGCAG-3′) and 1492R (5′-CGGTTACCTTGTAGCAGCTT-3′). The polymerase chain reaction (PCR) mixture consists of 30–50 ng DNA template, 2.5 mM dNTP mix, 0.5 μM primers, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 5U Taq polymerase. Amplification conditions were as follows: denaturation for 2 min. at 94 °C; 30 cycles of amplification; 40 s. denaturation at 94 °C; 45 s. annealing at 54 °C; 1 min. extension at 72 °C followed by final extension 10 min. at 72 °C. In case of *Trichoderma*, 18S rRNA gene (partial sequence), internal transcribed spacer 1, 5.8S rRNA gene internal transcribed spacer 2 (complete sequence) and 28S rRNA gene (partial sequence) were amplified by using the primer combinations ITS1 (5′-TCCGTA GGTGAAACCTTGGG-3′) and LR3R (5′-GGTCCGGTT TTCAGAC-3′) with fragment size of ~1200 bp; using the following conditions, viz., 1 min initial denaturation at 94 °C; 30 cycles of 1 min. denaturation at 94 °C; 1 min primer annealing at 50 °C; 90 s. extension at 74 °C and a final extension period of 7 min. at 74 °C. Size of the PCR amplicons were analysed on 1% agarose gel.
Purification of PCR product and sequencing
PCR amplicon fragments were purified using Promega Wizard® SV Gel and PCR Clean-Up System kit, and the purified products of about concentration 50–100 ng/ul were sequenced using Sangers sequencing method. The nucleotide sequences were submitted to NCBI GenBank database and NCBI accession numbers were obtained. These sequences were further aligned and compared with the sequences of *Bacillus* and *Trichoderma* species available with NCBI. NCBI BLAST homology searches of the respective gene sequences which performed to assess homologous sequences available in NCBI. Computational analysis of DNA sequence data, sequence editing, multisequence alignment and molecular phylogeny were performed using EMBL-EBI.

In vitro inhibition of Xoo by *Trichoderma* and *Bacillus* sp.
Inhibitory efficiency of *Trichoderma* on Xoo was analysed using dual culture competition-suppression assay (Sinclair and Dingra 2017). A loop of individual isolates *Trichoderma* mycelia along with spore and Xoo was placed exactly opposite to each other on a Petri plate maintaining equidistance from centre, containing modified Wakimoto media (MWM). Control plate was maintained by inoculating Xoo alone. Radial growth of Xoo was measured in treated as well as control plate and percent inhibition was calculated by the formula (Gangwar and Sinha 2010)

\[
\text{Percentage inhibition} = \frac{C - T}{C} \times 100
\]

where \(C\) = colony growth in centimetre in control plate, \(T\) = colony growth in centimetre in treated plate.

Efficacy of *Bacillus* on Xoo was established by slightly modifying the agar well diffusion technique (Sinclair and Dingra 2017). Four diffusion wells opposite sides to each other on PYEM plate containing one day old colony of Xoo were added with the broth of *Bacillus*. The suppression was observed as overgrowth of *Bacillus* isolates over Xoo. Quantitative estimation of *Bacillus* efficiency against Xoo was obtained by measuring the optical density \(\text{OD}_{600\text{nm}}\) of broth containing *Bacillus* and Xoo. *Bacillus* isolates were grown on LB broth at 37 °C in rotary shaker at 160 rpm for 48 h. Culture filtrates were obtained by centrifugation of broth containing culture @16,000 rpm for 10 min and filtered sterilized twice through 0.22 µm filter. Different concentrations (10, 25, 50, 75 and 100%) of culture filtrate were tested to finalize the lethal concentration for pathogen. In 20 ml of broth containing different concentrations of *Bacillus* filtrates, a 100 µl of Xoo broth culture was added and kept for incubation at 28±2° for 48 h. Negative control was maintained with Xoo alone in the LB broth (Elshakh et al. 2016). \(\text{OD}_{600\text{nm}}\) was measured after 72 h of incubation.

In vitro inhibition of *R. solani* by *Trichoderma* and *Bacillus* sp.
Antagonistic potential of both the antagonists against *R. solani* was studied using dual plate competition assay (Marzano et al. 2013). Five mm mycelial discs of both *Trichoderma* isolates and *R. solani* were placed opposite to each other equidistance from centre of a Petri plate containing PDA. The radial growth of *R. solani* with *Trichoderma* isolates was recorded along with control. The efficiency of *Bacillus* isolates on *R. solani* was estimated in the similar method as done for *Trichoderma* sp. with slight modification to make up for the differences in the rate of growth of the *Bacillus* when compared to *R. solani* (Huang et al. 2012). *R. solani* was grown for 24 h at a corner of a plate with NA, followed by streaking a loop of individual *Bacillus* at the opposite edge. Plates with pathogen alone served as control. The plates were incubated at room temperature for 2 days, and thereafter, the radial growth of *R. solani* was measured and recorded.

Percentage inhibition was calculated for both the protocol using above-mentioned formula (Gangwar and Sinha 2010).

Compatibility of consortia mixtures
Isolates of *Trichoderma* and *Bacillus* were individually tested against each other for their compatibility (Siddiqui and Shaukat, 2003). Different isolates of *Bacillus* grown separately on PYEM plates were streaked perpendicular to each other on a fresh plate containing 20 ml PYEM. Similarly, 5-mm disc from 7-day-old cultures of the isolates of *Trichoderma* grown separately was placed in a fresh plate containing PDA, maintaining equal distance with each other. Both the consortia were incubated at 27±2 °C (Fig. 1). Zone of inhibition, if any formed was measured as the incompatibility against the two antagonists. The test was replicated multiple times.

Mass multiplication of antagonists for seed and soil application
Antagonists slurry made from 4-day-old cultures was suspended in sterile water to make a uniform suspension and used for seed treatments. For soil application, the antagonists were cultured in their respective broths. About 100 ml of broth (\(\times 10^6\) and \(\times 10^4\) CFU/ml, respectively, for *Bacillus* and *Trichoderma*) was mixed with 1 kg of powdered rice bran and carboxymethyl cellulose (CMC) @2 g/Kg. This mixture was used @100 g per pot as per the treatment schedule. Consortia of compatible microbes were formulated as above by mixing both the antagonists in their respective seed and soil formulations.
in equal proportions. In all the above formulations, the inoculum density of $2.14 \times 10^7$/ml ($OD_{600nm} \sim 1.0$) for *Trichoderma* and $1.08 \times 10^8$/ml ($OD_{600nm} \sim 0.4$) for *Bacillus* were maintained in the liquid suspension (Beal et al. 2020).

### In vivo screening of isolated antagonists against *Xoo* and *R. solani* in net-house

The experiments were conducted under controlled conditions in net-house. The seeds of susceptible rice cultivar TN were soaked in sterile water for 24 h and after draining the excess water, were mixed with the antagonist suspension (@10 ml/Kg seeds) and incubated for 12 h. Treated seeds were then kept on blotting paper to test the germination percentage. About 25 seeds from the initial lot were then placed in nursery trays and monitored for 10 days to calculate morphological parameters like seedling length, seedling dry weight, vigour index-1 (germination % X seedling length) and vigour index-2 (germination % X seedling dry weight). About 25-day-old seedlings from the nursery were transplanted in pots of size 30 × 25 cm with about 5–7 kg of soil. Thirty days after transplanting (DAT), antagonists were applied to soil @ 10 g/Kg of soil. The pathogens were inoculated at 40 DAT. *Xoo* was inoculated by leaf clipping method by diluting with 10 mM MgCl$_2$ and maintaining the 0.5 $OD_{600}$ (Ke et al. 2017). Inoculation of *R. solani* was done by placing about 0.5 mg of sclerotia in rice sheath and covering it by moist cotton swab (Singh et al. 2002). The experiments were repeated during 2 seasons with 3 replications under controlled conditions in net-house and the values were averaged.

### Statistical analysis

The experiments were conducted in completely randomized design (CRD) and data were subjected for one-way analysis of variance (ANOVA), using post hoc test with Duncan's multiple range test (DMRT) at 5% ($P \leq 0.05$) significance level in SPSS 20.0.1 version. Correlation analysis and graphs were made using Microsoft excel 2019. Three replications were maintained during each experiment.

### Results

#### Isolation and identification of isolated pathogens

The *Trichoderma* and *Bacillus* isolates collected from rice rhizosphere samples, one potential isolate of *Trichoderma*, viz., T7 and 3 isolates of *Bacillus*, viz., B2, B3 and B4, were considered for further studies on the basis of the growth rate, colony morphology and in vitro screening against the pathogens. Along with these selected isolates, 4 potential isolates of *Trichoderma*, viz., TAIK 1 (*T. asperellum* IIRRCK1), TAIK 2 (*T. asperellum* IIRRCK2), TAIK 3 (*T. asperellum* IIRRCK3) and TAIK 4 (*T. asperellum* IIRRCK4) obtained from the Department of Plant Pathology, Hyderabad, were used in the studies. The morphological features of the selected *Trichoderma* and *Bacillus* isolates were described (Tables 1, 2). The microscopic features of TAIK 1 to 4 isolates have been described earlier in detail by Kannan et al. (2018). The key microscopic features of T7 were oval/round-shaped conidia, aggregated and irregularly branched conidiophores fertile at the tip, phialides were sigmoid, sparingly produced from the main axis and chlamydospores typically

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**Fig. 1** Compatibility studies among the individual consortia of selected fungal and bacterial isolates. Note: Picture represents the consortia compatibility test among bioagents. **A:** Consortia of five *Trichoderma* isolates {a: TAIK1, b: TAIK2, c: TAIK4, d: TAIK5 and e: TAIK3} on PDA media **B:** Consortia of *Bacillus* {a: BIK2, b: BIK3, c: BIK4} isolates on PYEM. Compatibility of the isolates with each other is confirmed with no inhibition zone.
## Table 1  Collection and identification of the isolated fungal antagonists

| Isolate name | Scientific name | Place of collection (latitude/longitude) | NCBI accession number | Colony morphology in standard PDA medium | Sporulation | Colour of spores | Days for maturation |
|--------------|----------------|------------------------------------------|-----------------------|------------------------------------------|-------------|------------------|---------------------|
| TAIK1        | *Trichoderma asperellum* | Hyderabad (17.3220°N, 78.4023°E) | MH825714 | Dark green | 3.7 ± 0.12 | Smooth mat with concentric rings | Yellowish Green | 4 |
| TAIK2        | *Trichoderma asperellum* | Hazaribagh (23.9925°N, 85.3637°E) | MH825715 | Yellowish green | 3.2 ± 0.08 | Smooth mat | Light green | 3 |
| TAIK3        | *Trichoderma asperellum* | Raipur (21.2514°N, 81.6296°E) | MH825716 | Light green | 3.0 ± 0.03 | Smooth mat | Light green | 3 |
| TAIK4        | *Trichoderma asperellum* | Rewa (24.5362°N, 81.3037°E) | MH825717 | Dark green | 4.1 ± 0.10 | Fluffy mat | Dark green | 3 |
| TAIK5        | *Trichoderma asperellum* | Hyderabad (17.3220°N, 78.4023°E) | MT802436 | Dark green | 4.5 ± 0.04 | Smooth mat | Dark green | 2 |

## Table 2  Collection and identification of the isolated bacterial antagonists

| Isolate code | Scientific name | Place of collection (latitude/longitude) | NCBI accession number | Colony morphology in PYEM | Colour of spores | Texture |
|--------------|----------------|------------------------------------------|-----------------------|--------------------------|------------------|---------|
| BIK2         | *B. velezensis* | Karimnagar (18.4386°N, 79.1288°E) | MW181655 | Grey white | 1.5 ± 0.03 | Round, smooth and moist |
| BIK3         | *B. subtilis*  | Hyderabad (17.3220°N, 78.4023°E)     | MW181668 | Off-white | 2.1 ± 0.10 | Flat, opaque and dry |
| BIK4         | *B. paralicheniformis* | Nalgonda (17.0575°N, 79.2684°E) | MW180949 | Pinkish white | 1.8 ± 0.09 | Irregular and extra slimy |
absent. *Bacillus* isolates B 1 to 3 in were gram-positive, rod-shaped, spore-forming bacteria varying in size. However, they differed in the colony colour, viz., B2 was grey white, B3 was off-white, and B4 was pinkish white. SEM images obtained revealed that conidia of TAIK 1 were warded (0.02 µm), oval in shape (2.64 µm) with aggregated branched conidiophore (15.7 × 2.25 µm) and bottle-shaped clustered phialides. B3 colonies were rod-shaped, atrichous, with a size of 1.38 × 0.75 µm (Table 3).

**Sequence-based identity of *Bacillus* and *Trichoderma* isolates**

In addition to the morphological characterization of the experimental *Bacillus* isolates, amplification of 16S rRNA and endoglucanase regions using specific primers

| Organism | Compound microscope images | Scanning Electron Microscope images |
|----------|----------------------------|-----------------------------------|
| TAIK1    | ![TAIK1 40X](image)        | ![TAIK1 SEM 5000X](image)         |
|          | ![TAIK1 100X](image)       | ![TAIK1 SEM 5000X](image)         |
| BIK3     | ![BIK3 100X](image)        | ![BIK3 SEM 5000X](image)         |

**Fig. 2** Amplification pattern of bacterial and fungal strains with ITS (fungal specific), 16S rRNA (bacterial specific) and endoglucanase (*Bacillus subtilis* specific)
(Fig. 2a, b) and their alignment against related sequences in NCBI revealed a similarity index of 97–98% for B2 with \textit{B. velezensis} (Fig. 3), 96–97% for B3 with \textit{B. subtilis} (Fig. 4) and 96% for B4 with \textit{B. paralicheniformis} (Fig. 5). They were named as BIK 2 (\textit{B. velezensis} IIRRCKB2), BIK 3 (\textit{B. subtilis} IIRRCKB3) and BIK 4 (\textit{B. paralicheniformis} IIRRCKB4). Phylogenetic analysis within the species indicated that the two isolates, viz., BIK 2 and BIK 3, were closely related with a sequence homology of 98.6%, while they were 92–93% with BIK 4. It can thus be concluded that BIK 2 and BIK 3 are genetically closer in comparison with BIK 4 isolate. In case of T7, the ITS region sequence alignment in the NCBI database indicated 98–99% similarity with \textit{T. asperellum} and named as TAIK 5 (\textit{T. asperellum} IIRRCK5) (Figs. 2c and 6). In order to differentiate with other \textit{T. asperellum} isolates from our study (Kannan et al. 2018), all the nucleotide sequences were aligned (TAIK 1, TAIK 2, TAIK 3 and TAIK 4 with TAIK 5) and results revealed a sequence homology of 98–99% among the isolates, indicating genetic similarity within the isolates. The nucleotide differences among the \textit{Bacillus} and \textit{Trichoderma} strains have also been analysed (Fig. 7a and b, respectively).

**Growth inhibition of Xoo and \textit{R. solani} by \textit{Trichoderma} and \textit{Bacillus} sp. in vitro**

Significant reductions in the growth rates of \textit{Xoo} and \textit{R. solani} over control with different \textit{Trichoderma} isolates were observed initially in the dual culture plates and in later days of observation, the plates were completely covered by \textit{Trichoderma} and an overgrowth of \textit{Trichoderma} on Xoo colonies was observed. Among different isolates of \textit{Trichoderma} and \textit{Bacillus}, TAIK 1 was found to be significantly effective in inhibiting the growth of both the pathogens (Figs. 8a,c, 9a). Among \textit{Bacillus} isolates, complete growth of BIK 3 upon Xoo was observed after 48 h (Fig. 8b) and the cultural filtrate (concentration 75%) obtained from BIK 3 was the most efficient in reducing
the viable population of pathogen as compared with other cultures (Fig. 9b.A). The *Bacillus* isolates were also found to suppress *R. solani* and the inhibition zone and percentage of growth reduction was comparatively high in BIK 3 (Figs. 8d, 9b.B).

**Plant growth promoting activities of Trichoderma and Bacillus isolates**

All the cultures, in general, showed significant improvement in seed germination and further growth of rice seedlings. Based on initial 10 days parameters of seed germination and growth, vigour index-1 and vigour index-2 were found to be the highest in the consortia treatment of *Trichoderma* isolates. Further, TAIK 1 was found to be significantly better than the combined effect of *Bacillus* consortia. A similar trend was observed in the case of root and shoot lengths of the seedlings studied after 25 DAS (Table 4 and Fig. 10).

**In vivo screening of Trichoderma and Bacillus isolates against Xoo and R. solani**

Results obtained from the disease scoring using standard evaluation system (SES) under glass house
conditions indicated that the plants treated with the consortia of *Trichoderma* isolates had significantly less scores of sheath blight disease and the diseases scores of BB were significantly lesser in the plants treated with consortia of *Bacillus* isolates than other treatments. A comparative analysis between the progresses of lesion length and root/shoot length showed inverse relationship for each of the treatments (Fig. 11). Further among the 2 most potential antagonists studied here, it was observed that TAIK 1 was more effective in improving plant growth than BIK 3.

**Discussion**

Biological control offers a viable alternative to the chemical management strategy for disease with no or very less genetic resistance in host plants (Ahemad and Kibret...
However, for biocontrol strategy to be effective, the most important criteria are to find potential strains of antagonists that effectively suppress the pathogen under a broad set of environment and soil conditions, survive competitively in the introduced target areas and improve the overall development and yield of the crop (Pieterse et al. 2014). The present studies conclusively demonstrated the efficiency of native isolates of two major genera of bioagents, viz., *Trichoderma* and *Bacillus* against the two rice pathogens tested, viz., *R. solani* and *Xoo*. Both bioagents are well known for their antagonistic potential and plant growth promoting activities. Confirmation through molecular analysis compliments the conventional morphological and biochemical techniques. Molecular identification of microbial community requires specific primers for sequencing so as to get more accurate and precise results than the use of universal primers (Janda and Abbott 2007). In recent years, molecular diversity studies have changed the taxonomic classification of bacterial and fungal isolates. There are various other fungal micro-organisms which have been identified with the help of ITS sequencing technique (Lieckfeldt et al. 1999). *Bacillus* isolates were identified based on amplification of endoglucanase region of the 16S rRNA, which is specific to directly identify particular species from ‘B. subtilis’ group’ (Mukherjee et al. 2017). Phylogenetic tree was constructed based on 16S rRNA and 18S rRNA gene sequence analysis of both bacterial and fungal antagonists, respectively. Sequence similarity within the isolates at nucleotide sequences level revealed differences within the isolates. However, unusual similarities exist for members of the ‘Bacillus 16S rRNA group’, including *B. subtilis*, which displays 99.3% similarity at the 16S rRNA level to *B. atropaeus* and 98.3% to *B. licheniformis* and *B. amyloliquifaciens* (Ash et al. 1991). Thus, in order to be specific, endoglucanase specific primers were used and 2 isolates (BIK 2 and BIK 3)
among 3 Bacillus were found to be positive for endo-
glucanase, while the other were negative. But, sequence
similarity index showed 98% identity with B. velezensis
(BIK 2). These set of samples shall be further character-
ized using whole genome sequencing by this group.

Results from the dual plate assay indicated the decrease
in radial growth of Xoo before coming in direct contact of
Trichoderma isolates which could be because of release of
antimicrobial compounds by Trichoderma in the
medium. Several antimicrobial compounds, both volatile
and non-volatile, have been identified from Trichoderma
and were established to be effective against various plant
pathogens in different crops (Reino et al. 2008). How-
ever, the direct growth of Trichoderma upon Xoo and
the resulting reduction in the number of colonies of Xoo
is an effect of utilization of the nutrients from the dead
colonies of the bacteria, which were killed by the anti-
microbial compounds of Trichoderma. Saprophytic growth
of Trichoderma species has been well established (Stef-
fan et al. 2020). In the case of R. solani, dual culture
plate assay indicated that Trichoderma was able to para-
sitize the pathogen mycelia in a very aggressive manner
and the growth of R. solani was severely inhibited. The
sclerotia were also colonized and lost their ability to ger-
minate effectively. Trichoderma colonizes, Rhizoctonia
by means of release of cell wall degrading enzymes that
helps them to penetrate inside the cell. Once inside the
cell, Trichoderma engulfs the cell contents by convert-
ning complex molecules into simpler substances (Halifu
et al. 2020). In in vitro assay of Bacillus against R. solani,
the zone of inhibition exhibited by the colonies of Bacil-
lus spp. confirmed the production of antibiotics, and in
the later period they lose their original shape and texture.
This denaturing effect of Bacillus on other pathogens was
reported earlier (Huang et al. 2012). Cultural filtrate stud-
ies conducted against Xoo also showed the appropriate
release and efficiency of bioactive compounds by Bacil-
lus isolates. Co-cultivation studies with transmission
electron microscopy analysis indicated concentration of
Bacillus inside the cytoplasm of Xanthomonas leading to
altered surface morphology resulted in the leakage and
further shrinkage of the cells (Xie et al. 2018).

Earlier studies by this group on the key role of Tricho-
derma spp. in increasing the germination percentage,
seedling length and seedling dry weight of rice were
found to be by direct production of growth regulating
hormones (Chinnaswami et al. 2021). Similarly, mem-
ers of Bacillus were reported to induce cytokinin, a
cell division promoting growth related hormone, which
was found to enhance the seedling growth and develop-
ment (Arkhipova et al. 2005). Growth improvement in
seedlings may also be due to the increase in production
of amylase (exogenous modulators) which hydrolyses the
starch into simple sugars and in turn provides energy for
growth of roots and shoots in germinating seedlings. Bio-
control activities of both the bioagents tend to stimulate
defence system in plants, which includes production of
PR proteins, phytoalexins and activation of induced sys-
temic resistance (ISR) by synthesis of jasmonic acid, eth-
ylene and NPR-1 regulatory gene (Konappa et al. 2020).

Conclusions
Several antagonistic bacteria and fungi belonging to the
genus Bacillus and Trichoderma were isolated from the
native rice soils from in and around Hyderabad, Tel-
angana. The isolates were screened in vitro for their
antagonistic efficiency and the selected potential iso-
lates were carried forward for further identification and
antagonistic studies tested against the two major rice
pathogens, viz., R. solani and Xoo. Identification based
on morphological characters were confirmed using
molecular tools. Accordingly, phylogenetic tools based
on 16srRNA and ITS gene sequences were used for
identifying the isolates of Trichoderma and Bacillus
at species level. The diversity analysis was suitable alterna-
tive method to phenotypic procedures for reliable iden-
tification of unknown isolates at species level or at least
useful in the primary differentiation at species level
from those of other groups. The individual isolates of
the 2 antagonists varied in their efficiency to suppress
the pathogens and promote plant growth. Consortia
of the isolates were found to be more effective than
the individual ones. Among the different isolates, the
results obtained indicated the highest antagonistic effi-
ciency of two native isolates TAIK 1 and BIK 3 against
the two major pathogens of rice. In addition, the bioag-
ents also enhanced the root and shoot growth of rice,
indicating a health plant growth to defend against the
pathogens.

(See figure on next page.)

**Fig. 7** a Nucleotide alignment of sequences in Bacillus strains. Nucleotide differences and insertions are highlighted. Note: The sample IDs 2CK, 3CK and 4CK represent BIK2, BIK3 and BIK4 strains, respectively. b Nucleotide alignment of sequences in Trichoderma strains. Nucleotide differences and insertions are highlighted. The sample IDs CK2-T-2, CKTV-1, CK1-T-1, CK3-T-3 and CK4-T-4 represent TAIK2, TAIK5, TAIK1, TAIK3 and TAIK4 strains, respectively.
Fig. 8  In vitro efficiency of bioagents against Xoo and R. solani: a Antagonism of TAIK1 on Xoo in MWM (left) and Xoo control, b overgrowth of BIK3 upon Xoo in MWM (left) and Xoo control, c antagonism of TAIK1 on R. solani in PDA (left) and R. solani control, d antagonism of BIK3 on R. solani in PDA and R. solani control.

Fig. 1a

Fig. 1b

Fig. 9  a In vitro screening of different isolates of Trichoderma spp. against Xoo and R. solani. Data represent mean of three replications in each treatment. X-axis represents the five isolates of Trichoderma taken for the dual culture assay. b In vitro screening of different isolates of Bacillus spp. against Xoo and R. solani. Data represent mean of three replications in each treatment. A OD$_{600nm}$ values of sample containing culture filtrate (75% concentration) of different Bacillus isolates incubated with Xoo for 72 h. B Percent inhibition of R. solani by Bacillus isolates after 72 h of incubation.
Table 4 Screening of PGPR activities of isolated antagonists in TN1

| Isolates                     | Root length (cm) 25th DAS | Shoot length (cm) 25th DAS | Germinations | Vigour index-1 (10th day after germination) | Vigour index-2 (10th day after germination) |
|-----------------------------|---------------------------|----------------------------|--------------|--------------------------------------------|-------------------------------------------|
| TAIK1                       | 9.90<sup>b</sup>         | 13.20<sup>bc</sup>         | 100          | 610.33<sup>i</sup>                         | 17.00<sup>ab</sup>                        |
| TAIK2                       | 7.50<sup>ab</sup>         | 11.07<sup>abc</sup>         | 92           | 378.67<sup>bc</sup>                         | 10.31<sup>b</sup>                         |
| TAIK3                       | 7.53<sup>ab</sup>         | 10.27<sup>abc</sup>         | 90           | 404.77<sup>c</sup>                         | 11.19<sup>bc</sup>                        |
| TAIK4                       | 8.57<sup>ab</sup>         | 12.23<sup>bc</sup>         | 92           | 460.00<sup>e</sup>                         | 12.24<sup>bcd</sup>                       |
| TAIK5                       | 8.83<sup>ab</sup>         | 12.47<sup>bc</sup>         | 98           | 539.00<sup>f</sup>                         | 13.25<sup>bcde</sup>                      |
| BIK2                        | 7.87<sup>ab</sup>         | 9.63<sup>ab</sup>          | 96           | 442.33<sup>d</sup>                         | 11.83<sup>bc</sup>                        |
| BIK3                        | 8.63<sup>ab</sup>         | 13.13<sup>bc</sup>         | 100          | 560.00<sup>g</sup>                         | 15.11<sup>bcd</sup>                       |
| BIK4                        | 7.97<sup>ab</sup>         | 10.47<sup>abc</sup>         | 98           | 470.00<sup>e</sup>                         | 13.09<sup>bcd</sup>                       |
| Trichoderma isolates consortia | 10.17<sup>b</sup>       | 14.07<sup>d</sup>          | 100          | 640.67<sup>i</sup>                         | 18.13<sup>bc</sup>                        |
| Bacillus isolates consortia  | 9.40<sup>ab</sup>         | 13.53<sup>bc</sup>         | 100          | 590.67<sup>h</sup>                         | 15.66<sup>bcde</sup>                      |
| Healthy control             | 6.00<sup>a</sup>          | 7.77<sup>a</sup>           | 84           | 312.00<sup>a</sup>                         | 5.44<sup>a</sup>                          |

Data represent mean of three replications. Numerical values with different letters are significantly different (P < 0.05, DMRT, SPSS).

Fig. 10 Changes in root length of plants treated with bioagents and inoculated with pathogens. Root length of TN1 after 20 days of Xoo (a) and R. solani (b) infection. TC: Trichoderma consortia, BC: Bacillus consortia, C: Control.

Abbreviations
BB: Bacterial blight; Xoo: Xanthomonas oryzae Pv. oryzae; R. solani: Rhizoctonia solani; ROS: Reactive oxygen species; ITS: Internal transcribed spacer; TSM: Trichoderma Specific medium; PYEM: Peptone yeast extract medium; SEM: Scanning electron microscopy; LB: Luria Bertani; PDB: Potato dextrose broth; PCR: Polymerase chain reaction; NCBI: National Center for Biotechnology Information; BLAST: Basic local alignment research tool; OD: Optical density; CMC: Carboxymethyl cellulose; H2O2: Hydrogen peroxide; APx: Ascorbate peroxidase; CAT: Catalase.

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Authors’ contributions
All authors have read and approved the manuscript. Conceptualization and review were done by C.K. Collection of materials and preparation of the draft were maintained by D.M. Editing of written text was done by G.R and S.K.H. P.M has assisted in conducting experiments. Editing of manuscript was done by R.M.S. All authors read and approved the final manuscript.

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Availability of data and materials
Data and materials in this study can be available on reasonable request.
Fig. 11 Screening of bacterial blight and sheath blight diseases of rice against isolated fungal and bacterial bioagents. Data represent the mean of three replications (*P*<0.05, Duncan's multiple range test, SPSS). CD (*P* = 0.05). The combo graph depicts relationship between disease progress (lesion length at 14th DAI and 21st DAI) and change in root and shoot length. a Bacterial blight. b Sheath blight. TC: Trichoderma consortia, BC: Bacillus consortia, PC: pathogen control.
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