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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 (Köhl et al. 2019). Development of resistant varieties,

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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 Bozzola 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 $8000 \times g$ 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′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-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 $MgCl_2$, 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 GGTGAACCTGCGG-3′) and LR3R (5′-GGTCCGTGT TTCAAGAC-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)

$$\text{Per cent inhibition (\%)} = \frac{C - T}{C} \times 100$$

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

Efficacy of f *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 (OD_{600nm}) of broth containing *Bacillus* and *Xoo*. *Bacillus* isolates were grown on LB broth at 37 °C in rotatory 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). OD_{600nm} 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

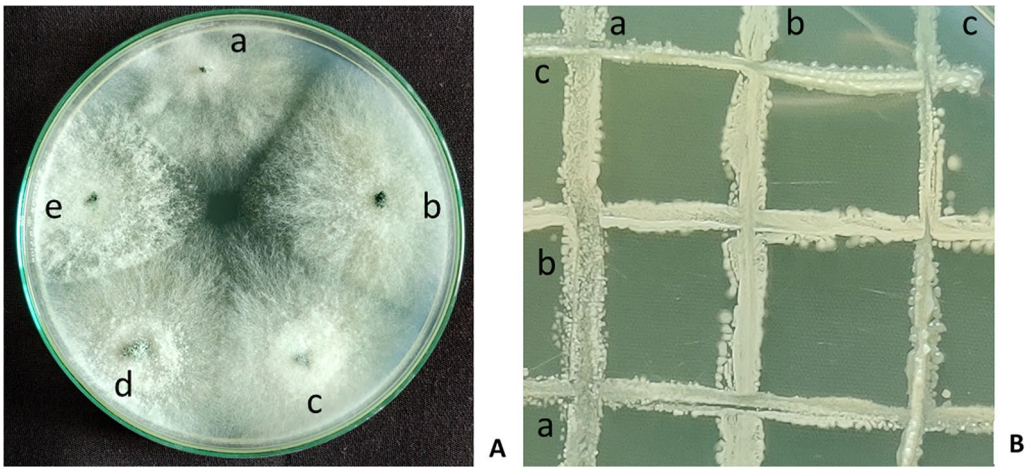


Fig. 1 Compatibility studies among the individual consortia of selected fungal and bacterial isolates. Note: Picture represents the consortia compatibility test among bioagents. **A:** Consortia of five *Trichoderma* isolates {**a**: TAIK1, **b**: TAIK2, **c:** TAIK4, **d:** TAIK5 and **e:** TAIK3} on 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

in equal proportions. In all the above formulations, the inoculum density of 2.14×10^7/ml ($OD_{600nm} \sim 1.0$) for *Trichoderma* and 1.08×10^8/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

Table 1 Collection and identification of the isolated fungal antagonists

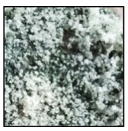
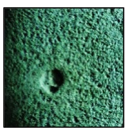
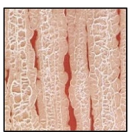
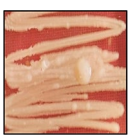
Isolate name	Scientific name	Place of collection (latitude/longitude)	NCBI accession number	Colony morphology in standard PDA medium				Sporulation	
				Colour	Colony	Radial growth of colony in 36 h (cm)	Texture	Colour of spores	Days for maturation
TAIK1	*Trichoderma asperellum* IIRRCK1	Hyderabad (17.3220°N, 78.4023°E)	MH825714	Dark green		3.7 ± 0.12	Smooth mat with concentric rings	Yellowish Green	4
TAIK2	*Trichoderma asperellum* IIRRCK2	Hazaribagh (23.9925° N, 85.3637° E)	MH825715	Yellowish green		3.2 ± 0.08	Smooth mat	Light green	3
TAIK3	*Trichoderma asperellum* IIRRCK3	Raipur (21.2514° N, 81.6296° E)	MH825716	Light green		3.0 ± 0.03	Smooth mat	Light green	3
TAIK4	*Trichoderma asperellum* IIRRCK4	Rewa (24.5362° N, 81.3037° E)	MH825717	Dark green		4.1 ± 0.10	Fluffy mat	Dark green	3
TAIK5	*Trichoderma asperellum* IIRRCK5	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

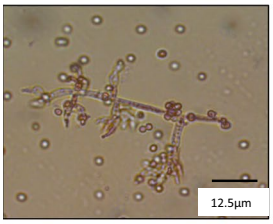
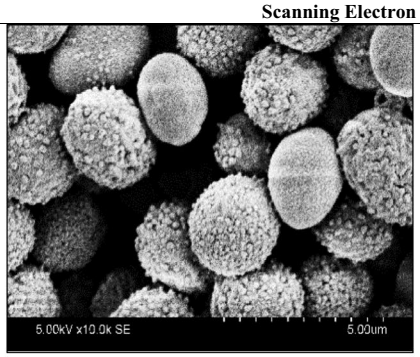
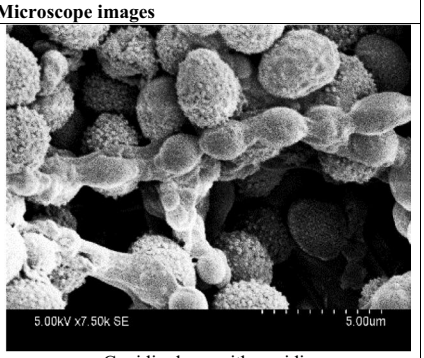
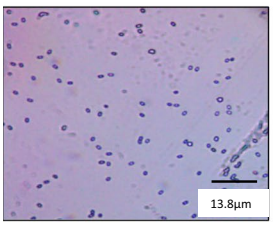
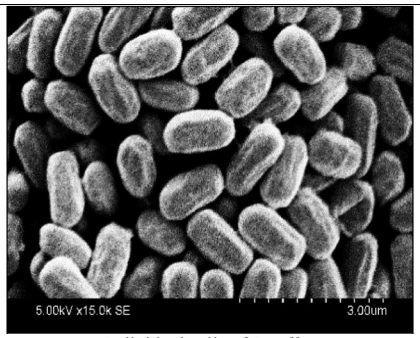
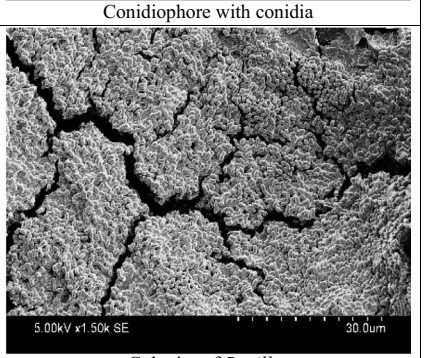
Isolate code	Scientific name	Place of collection (latitude/longitude)	NCBI accession number	Colony morphology in PYEM			
				Colour	Colony	Radial colony growth in 36 h (cm)	Texture
BIK2	*B. velezensis* IIRRCKB2	Karimnagar (18.4386° N, 79.1288° E)	MW181655	Grey white		1.5 ± 0.03	Round, smooth and moist
BIK3	*B. subtilis* IIRRCKB3	Hyderabad (17.3220°N, 78.4023°E)	MW181668	Off-white		2.1 ± 0.10	Flat, opaque and dry
BIK4	*B. paralicheniformis* IIRRCKB4	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 warted (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

Table 3 Microscopic features of the two potential antagonists

Organism	Compound microscope images	Scanning Electron Microscope images	
TAIK1	 (40X)	 Warted conidia	 Conidiophore with conidia
BIK3	 (100X)	 Individual cells of *Bacillus*	 Colonies of *Bacillus*

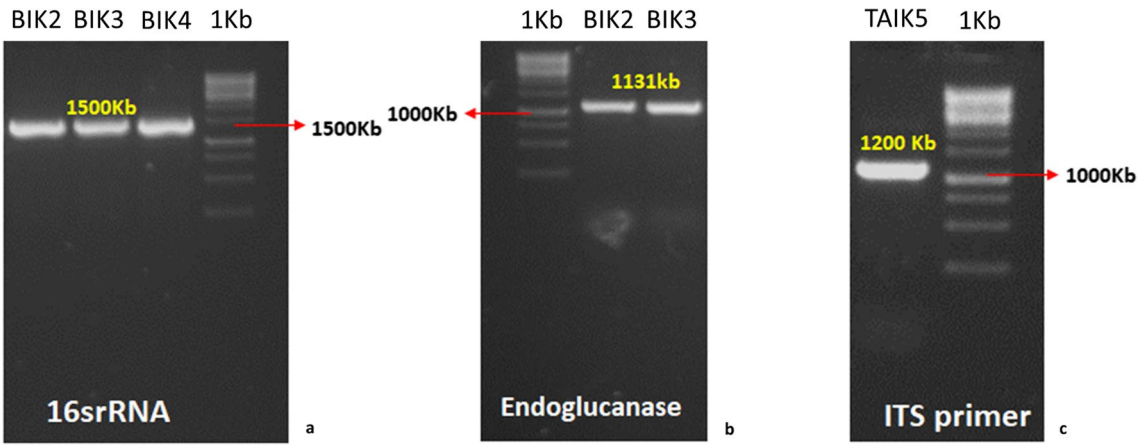


Fig. 2 Amplification pattern of bacterial and fungal strains with ITS (fungal specific); 16S rRNA (bacterial specific) and endoglucanase (*Bacillus subtilis* specific)

Fig. 3 Phylogenetic tree of 16S rRNA gene sequences for BIK2 strain constructed with related isolates from NCBI database as on December 2020

(Fig. 2a, b) and their alignment against related sequences in NCBI revealed a similarity index of 97–98% for B2 with *B. velezensis* (Fig. 3), 96–97% for B3 with *B. subtilis* (Fig. 4) and 96% for B4 with *B. paralicheniformis* (Fig. 5). They were named as BIK 2 (*B. velezensis* IIRRCKB2), BIK 3 (*B. subtilis* IIRRCKB3) and BIK 4 (*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 *T. asperellum* and named as TAIK 5 (*T. asperellum* IIRRCK5) (Figs. 2c and 6). In order to differentiate with other *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 *Bacillus* and *Trichoderma* strains have also been analysed (Fig. 7a and b, respectively).

Growth inhibition of *Xoo* and *R. solani* by *Trichoderma* and *Bacillus* sp. in vitro

Significant reductions in the growth rates of *Xoo* and *R. solani* over control with different *Trichoderma* isolates were observed initially in the dual culture plates and in later days of observation, the plates were completely covered by *Trichoderma* and an overgrowth of *Trichoderma* on *Xoo* colonies was observed. Among different isolates of *Trichoderma* and *Bacillus*, TAIK 1 was found to be significantly effective in inhibiting the growth of both the pathogens (Figs. 8a,c, 9a). Among *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

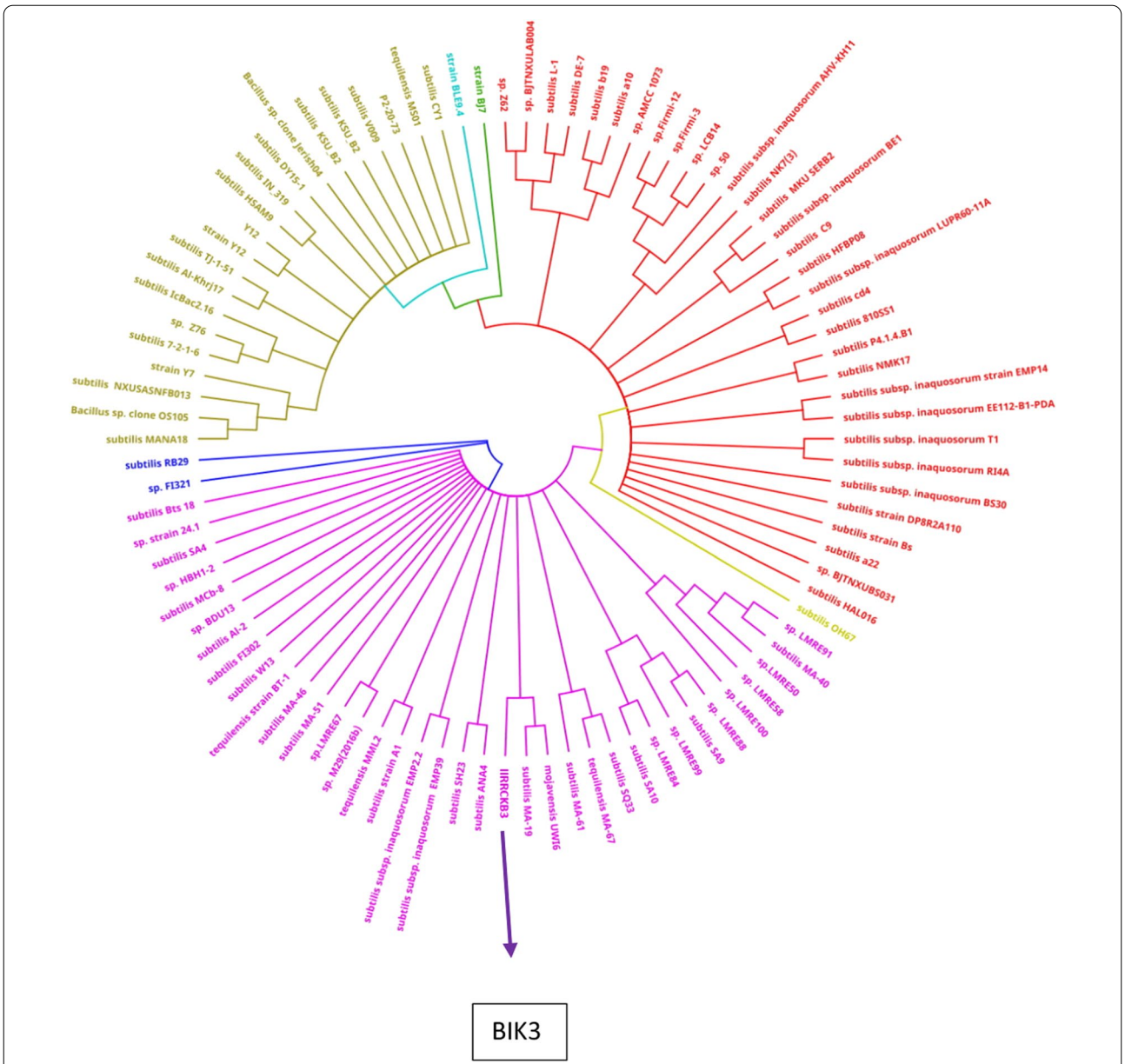


Fig. 4 Phylogenetic tree of 16S rRNA gene sequences for BIK3 strain constructed with related isolates from NCBI database as on December 2020

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

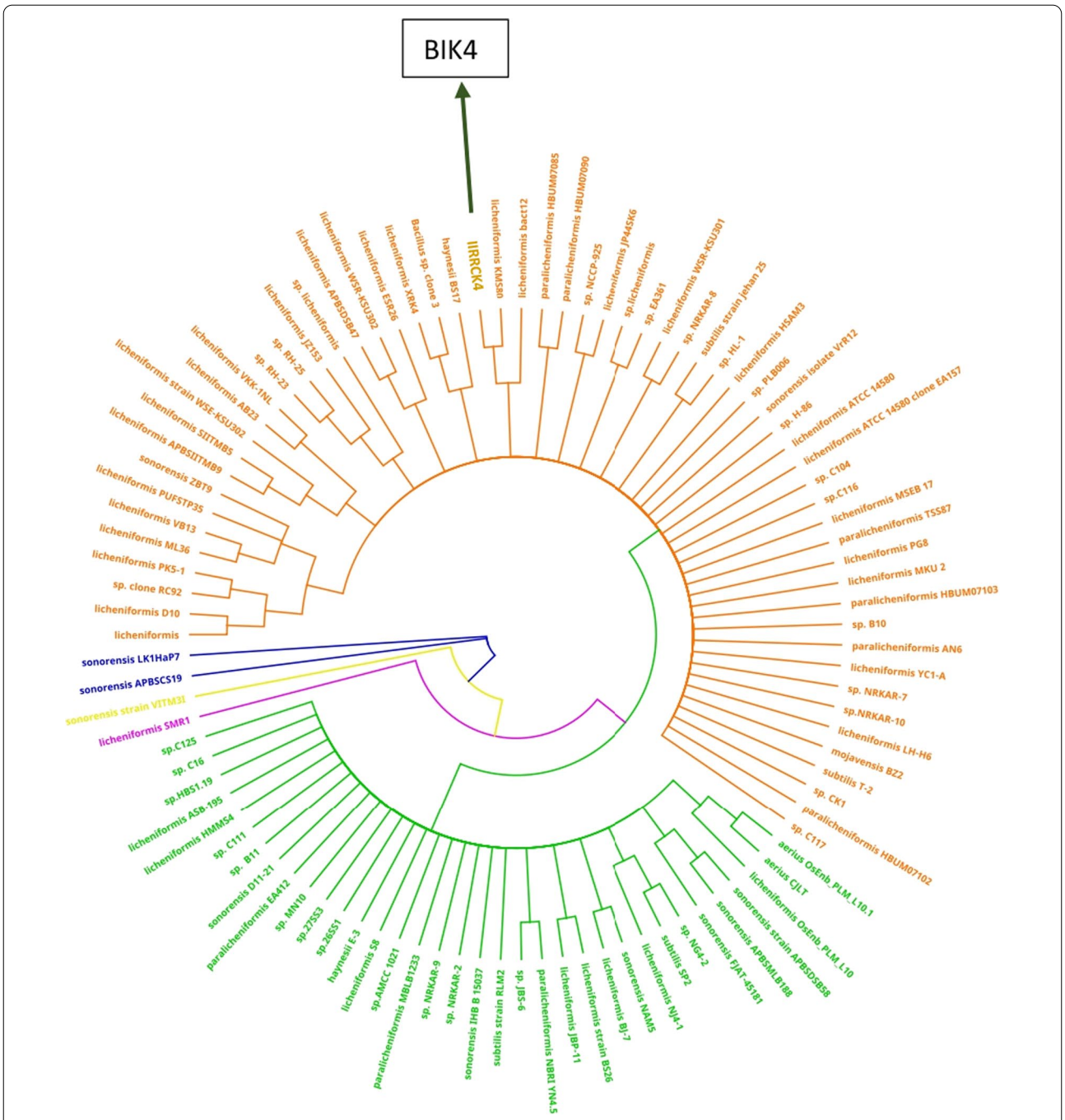


Fig. 5 Phylogenetic tree of 16S rRNA gene sequences for BIK4 strain constructed with related isolates from NCBI database as on December 2020

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

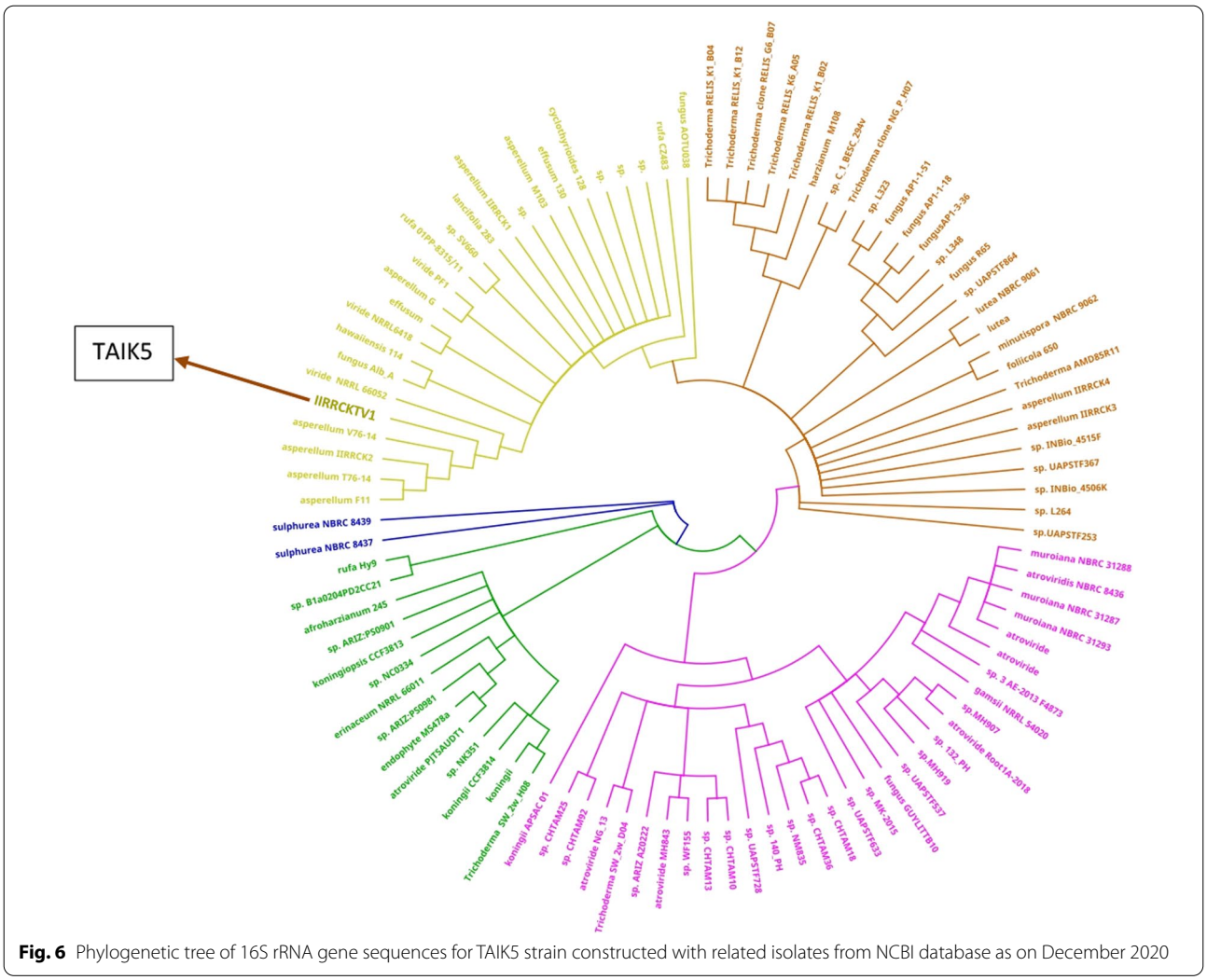


Fig. 6 Phylogenetic tree of 16S rRNA gene sequences for TAIK5 strain constructed with related isolates from NCBI database as on December 2020

2014). 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 I', including *B. subtilis,* which displays 99.3% similarity at the 16S rRNA level to *B. atrophaeus* and 98.3% to *B. licheniformis* and *B. amyloliquefaciens* (Ash et al. 1991). Thus, in order to be specific, endoglucanase specific primers were used and 2 isolates (BIK 2 and BIK 3)

(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

among 3 *Bacillus* were found to be positive for endoglucanase, while the other were negative. But, sequence similarity index showed 98% identity with *B. velezensis* (BIK 2). These set of samples shall be further characterized 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). However, 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 antimicrobial compounds of *Trichoderma.* Saprophytic growth of *Trichoderma* species has been well established (Steffan et al. 2020). In the case of *R. solani*, dual culture plate assay indicated that *Trichoderma* was able to parasitize 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 germinate 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 converting 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 *Bacillus* 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 studies conducted against *Xoo* also showed the appropriate release and efficiency of bioactive compounds by *Bacillus* 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 *Trichoderma* 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, members of *Bacillus* were reported to induce cytokinin, a cell division promoting growth related hormone, which was found to enhance the seedling growth and development (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. Biocontrol activities of both the bioagents tend to stimulate defence system in plants, which includes production of PR proteins, phytoalexins and activation of induced systemic resistance (ISR) by synthesis of jasmonic acid, ethylene 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, Telangana. The isolates were screened in vitro for their antagonistic efficiency and the selected potential isolates 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 alternative method to phenotypic procedures for reliable identification 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 efficiency of two native isolates TAIK 1 and BIK 3 against the two major pathogens of rice. In addition, the bioagents also enhanced the root and shoot growth of rice, indicating a health plant growth to defend against the pathogens.

Fig. 7 (See legend on previous page.)

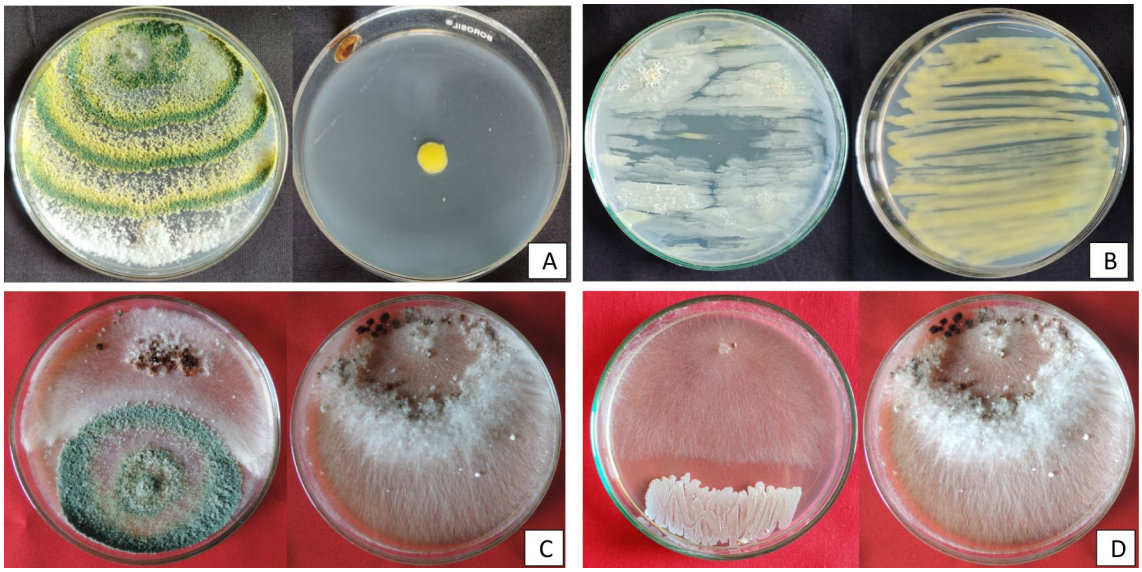


Fig. 8 In vitro efficiency of bioagents against *Xoo* and *R. solani*. **a** Antagonism of TAIK1 on *Xoo* in MWM (left) and *Xoo* control, **b** overgrowth of BIK3 upon *Xoo* in MWM (left) and *Xoo* control, **c** antagonism of TAIK1 on *R. solani* in PDA (left) and *R. solani* control, **d** antagonism of BIK3 on *R. solani* in PDA and *R. solani* control

Fig. 1a

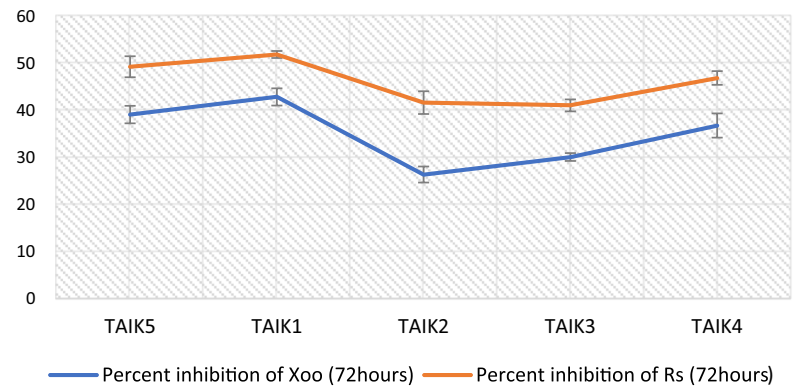


Fig. 1b

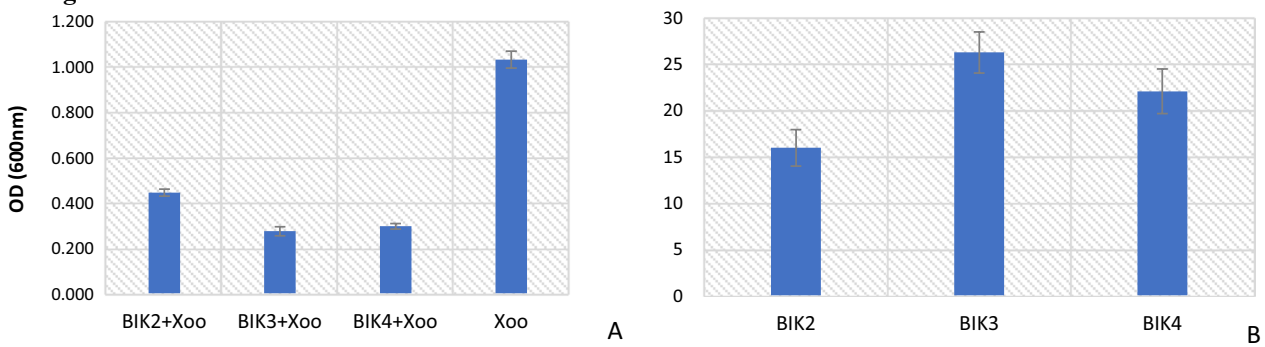


Fig. 9 **a**: In vitro screening of different isolates of *Trichoderma* spp. against *Xoo* and *R. solani*. Data represent mean of three replications in each treatment. X-axis represents the five isolates of *Trichoderma* taken for the dual culture assay. **b**: In vitro screening of different isolates 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^{b}	13.20^{bc}	100	610.33^{i}	17.00^{de}
TAIK2	7.50^{ab}	11.07^{abc}	92	378.67^{b}	10.31^{b}
TAIK3	7.53^{ab}	10.27^{abc}	90	404.77^{c}	11.19^{bc}
TAIK4	8.57^{ab}	12.23^{bc}	92	460.00^{e}	12.24^{bcd}
TAIK5	8.83^{ab}	12.47^{bc}	98	539.00^{f}	13.25^{bcde}
BIK2	7.87^{ab}	9.63^{ab}	96	442.33^{d}	11.83^{bc}
BIK3	8.63^{ab}	13.13^{bc}	100	560.00^{g}	15.11^{bcde}
BIK4	7.97^{ab}	10.47^{abc}	98	470.00^{e}	13.09^{bcd}
Trichoderma isolates consortia	10.17^{b}	14.07^{d}	100	640.67^{j}	18.13^{e}
Bacillus isolates consortia	9.40^{ab}	13.53^{bc}	100	590.67^{h}	15.66^{cde}
Healthy control	6.00^{a}	7.77^{a}	84	312.00^{a}	5.44^{a}

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

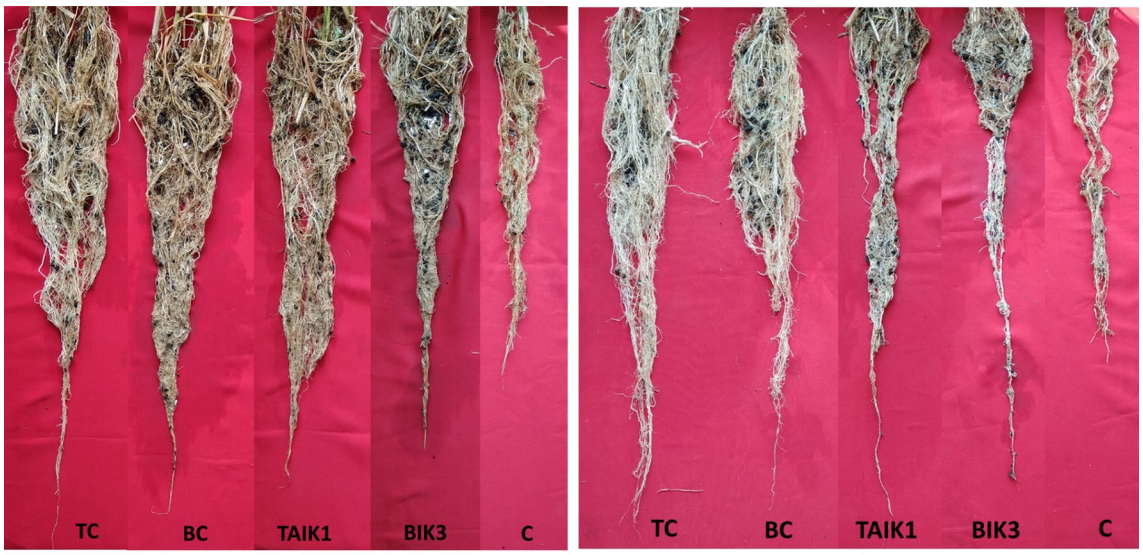


Fig. 10 Changes in root length of plants treated with bioagents and inoculated with pathogens. Root length of TN1 after 20 days of *Xoo* (**a**) and *R. solain* (**b**) infection. TC: *Trichoderma* consortia, BC: *Bacillus* consortia, C: Control

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; H_2O_2: 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 CK. Collection of materials and preparation of the draft were maintained by DM. Editing of written text was done by GR and SKH. PM has assisted in conducting experiments. Editing of manuscript was done by RMS. 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.

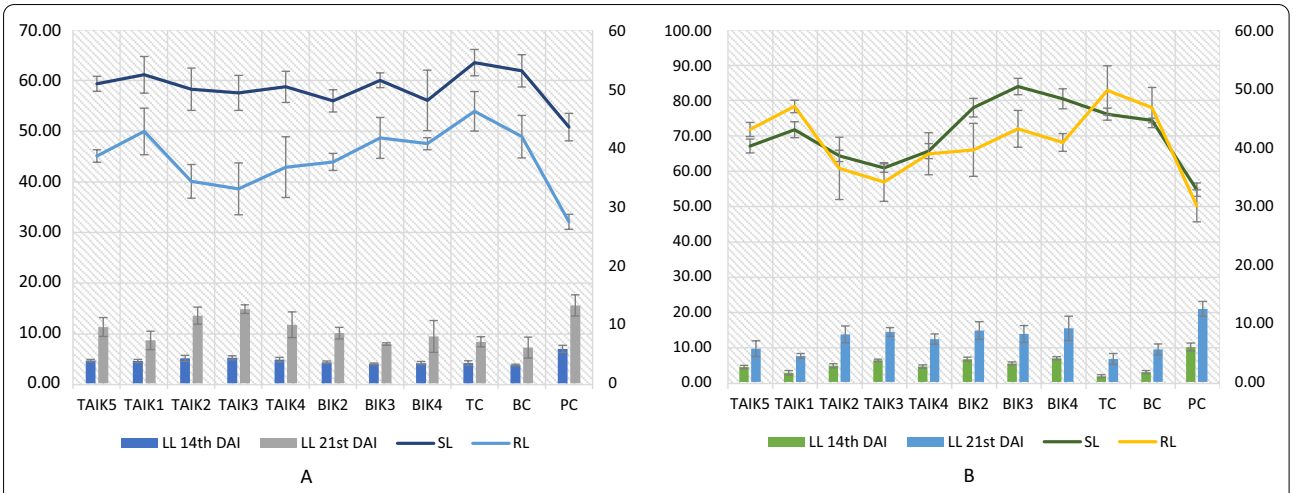

Fig. 11 Screening of bacterial blight and sheath blight diseases of rice against isolated fungal and bacterial bioagents. Data represent the mean of three replications ($P<0.05$, Duncan's multiple range test, SPSS). CD ($P=0.05$). The combo graph depicts relationship between disease progress (lesion 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

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No potential conflict of interest was reported by the author(s).

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