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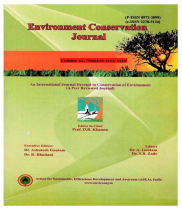

Friendly microbes help rice to grow and suppress its pathogens: *Trichoderma* and *Bacillus* Vs *Xanthomonas* in rice

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ABSTRACT

Use of biological control for the management of diseases has gained huge awareness and importance in the present situation of climate change and food residues. Biocontrol agents play interesting role in developing plant health and provide protection against biotic and abiotic stresses. In this study, we isolated *Trichoderma* and *Bacillus* sp. isolated from soil samples collected from rice fields in *Kharif* 2019. Profiling based on the pH of the soil, the fungal bioagents were more present in slightly acidic to neutral pH (5.8-7.2) whereas bacterial bioagents in slightly neutral to basic (7.4-8.3). The isolates were screened for their ability to produce phytohormones, cell-wall degrading enzyme and biofilm. Based on biochemical screening two *Trichoderma* isolates (T6 and T7) and two *Bacillus* isolates (B1and B5) were subjected to glasshouse studies. Per cent diseased leaf area and lesion length of plants treated with B1 were found to be effective against pathogen. However, the plant growth promotion was more enhanced by T6. Scanning electron microscopy and molecular characterisation along with their phylogenetic analysis proved the identity of isolate B1 as *Bacillus subtilis* and T6 as *Trichoderma atroviride.*

Introduction

Biological control in sustainable agriculture is providing promising contributions to manage the diseases and promote growth of the crop plants (O'Brien, 2017). It is the major element in integrated disease/pest management programme in agriculture in many countries (Ram *et al.,* 2018) and because of its stability and longevity, it is now considered as the primary method for disease/pest control. Biocontrol agents *viz., Trichoderma, Bacillus, Pseudomonas, Streptomyces* etc. are being utilized in agriculture and industrial sectors on large scale (O'Brien, 2017). They are used in production of biofertilizers, biopesticides and phytostimulators. They have excellent capacity to release growth promoting hormones which helps in the healthy development of plants (Doni *et al.,* 2014). They also possess strong antagonistic activities against various plant pathogens in different environmental conditions through direct mechanisms like hyperparasitism, antibiosis, competition of nutrients etc. and indirect mechanisms like systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse *et al.,* 2014; Singh *et al.,* 2019).

Rice being one of the most important food crops for more than half of the world's population, is continuously attacked by various biotic and abiotic stresses that leads to immense loss to crop (Hastuti *et al.,* 2012). Due to change in climate, genotypes

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grown and cultivation practices, the profile of the diseases in rice has changed over a period of time. Among the bacterial diseases, bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has caused tremendous yield loss to the farmers (Brunner *et al.,* 2005). Spraying of antibiotics is causing harmful residual effect on the soil. Though resistant varieties have gained popularity, but sudden breakdown of the resistance gene cause huge loss to the yield and income to the farmers. In order to complement the existing management strategies, biocontrol gives safe, sustainable and viable solution to the farmers to control the disease (Marin *et al.,* 2019).

The current experiment was conducted to isolate beneficial microbes from rice rhizosphere soil of Telangana state and study its characteristics to be a potential biocontrol agent against *Xoo.* The *Trichoderma* and *Bacillus* isolates were screened for their IAA, phosphate solubilisation, siderophore, cell-wall degrading enzymes and biofilm production capacity. They were tested for *in-vitro* for their capacity to inhibit growth of *Xoo* and on the basis of this results, potential bioagents were tested for their efficacy against *Xoo* under glasshouse conditions. The best fungal and bacterial isolates from *in-vivo* results were identified and the DNA sequence were submitted to NCBI.

Material and Methods

Soil samples were collected from different rice growing regions of Telangana state (Table 1) to isolate fungal and bacterial bioagents during *Kharif* 2019 (hot and humid climate: RH: 80%). At two vertical depths-15cm and 30 cm; the soil samples were taken randomly from field with the help of post- hole auger, air dried and taken for serial dilution. The pH of soil samples were recorded. Stock solution for all the samples were prepare by dissolving 1g of soil in 9ml of water. Samples were then diluted and fungal bioagents were isolated from dilution factor 10^{-4} by evenly spreading one ml of prepared dilution on Trichoderma specific medium (TSM). For bacterial bioagents (*Bacillus* sp.), stock soil sample were heated at 65^{o}C for 5min and then were isolated same as above in Luria Bertani agar (LBA) medium from dilution factor 10^{-8}. The plates were then incubated at 25±2^{o}C for 7 days for fungal and 2 days for bacterial bioagents. Morphological identification of fungal and bacterial bioagents were done based on colony structure in specific media (Istock, 2008; Gupta *et al.,* 2014). Microscopic identification of fungal bioagents were done by staining the sample with methylene blue and observing at 40X magnification in compound microscope. For bacterial bioagents, Gram staining was done and observed at 100X magnification in compound microscope. Fungal isolates were tested for their growth promoting activities by quantitative estimation of Indole acetic acid (IAA) (Gravel *et al.,* 2007), Phosphate solubilisation (Saravanakumar *et al.,* 2013), siderophore production (Payne, 1994), chitinase production (Miller, 1959) and β-1,3-gucanases (Ramada *et al.,* 2010) *in-vitro*.

Table 1: Rice rhizosphere samples from different locations of Telangana state

District	Mandal	Collection date	Soil type	Cropping pattern	Rice variety	Latitude	Longitude
Hyderabad	Rajendranagar	22-8-19	Black cotton soil	Rice-Rice	Samba Mahsuri (BPT-5204)	17.3220° N	78.4023° E
Khammam	Wyra	12-9-19	Red soil	Rice-Chilli	Telangana Sona (RNR-15048)	17.2473° N	80.1514° E
Khammam	Mittapalli	12-9-19	Red soil	Rice-Maize	Telangana Sona (RNR-15048)	17.2473° N	80.1514° E
Nalgonda	Chandupatla	19-9-19	Red soil	Rice-Rice	Samba Mahsuri (BPT-5204)	17.1883° N	79.2000° E
Nalgonda	Katangur	19-9-19	Black cotton soil	Rice-Rice	Samba Mahsuri (BPT-5204)	17.1883° N	79.2000° E
Nalgonda	Inupamula	19-9-19	Red soil	Rice-Maize	Telangana Sona (RNR-15048)	17.1883° N	79.2000° E
Suryapet	Husainabad	25-9-19	Red soil	Rice-Horse gram	Telangana Sona (RNR-15048)	17.1314° N	79.6336° E

Quantitative estimation of growth promoting parameters of bacterial bioagents included Indole acetic acid (IAA) (Brick *et al.,*1991), Phosphate solubilisation (Nautiyal, 1999), siderophore production (Schwyn and Neilands, 1987) and biofilm production (Yousef *et al.,* 2008) *in-vitro.* The pathogen was isolated from diseased leaf samples showing peculiar symptom of bacterial blight, collected from rice growing areas in Rajendranagar, Hyderabad. The diseased portion with healthy tissues was cut into 1.5 to 2 cm pieces and surface sterilized with 0.1 per cent sodium hypochlorite solution. These leaf pieces were further cut on microscopic slide with sterilized blade and upon that sterilized distilled water was dropped for preparation of bacterial suspension. This suspension was streaked on modified Wakimoto's agar (MWA) medium with the help of sterilized wire loop. The inoculated plates were incubated at room temperature (27 ± 2°C) for 48 hrs. The identification of *Xoo* was done with cultural morphology, gram staining, and 16s rRNA sequence analysis. Dual culture assay was performed in order to test the efficiency of isolated bioagents and pathogen. Ten *Trichoderma* and five *Bacillus* isolates were used for evaluating the suppression potential against *Xoo*. The media for dual culture was MWA as it allows both the bioagents to grow perfectly as it does for *Xoo*. Five mm bit from pure cultures of *Trichoderma* isolates were kept at one edge and *Xoo* at another edge in the petriplate maintaining equal distance from the centre. Control was maintained with only *Xoo* isolate. The radial growth of *Xoo* was measured in treated and control plate 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

For testing the antagonism efficiency of *Bacillus* isolates, the cultures were grown on Luria Bertani broth (LB) for 48 hrs and then the cultural filtrate (supernatant) was collected by centrifugation at 10000 rpm for 6 mins. The filtrate was passed through bacterial filter (0.22µm) 2 times so that no bacterial cells will remain in filtrate. This filtrate was then diluted by LB to make different concentrate (20, 40, 60, 80 and 100%) and tested against *Xoo* to find out minimum inhibitory concentration. Filter paper disc of 0.5cm radius were sterilised and dipped in different concentration of each bacterial isolate's filtrates. In the plate, first *Xoo* was streaked and then filter paper containing filtrate was placed at four places in the plate. Controls were maintained separately with no filtrate. Antibacterial activity was analysed by measuring the mean zone of inhibition diameters formed by individual filtrates (Balouiri *et al.,* 2016). Waste rice grains with husk were taken for mass multiplication of bioagents. The grains were coarsely grinded in mixer grinder kept in trays for bioagent application. Bioagents were grown in selective broth *viz*., Potato dextrose broth (PDB) and LB for *Trichoderma* and *Bacillus* isolates respectively. For *Trichoderma* isolates, the full-grown fungal mat with spores was separated with sterile forceps and crushed in mortar pestle to make slurry. This paste was poured in trays maintaining CFU @ 2.14 x 10^6 /ml and moisture content of 68.23 ± 0.07%. In case of *Bacillus* isolates, the culture in LB was shacked completely and poured in trays with CFU of 1.08 x 10^8 /ml and moisture content of 70.35 ± 0.02%. The mixtures were used for soil application @ 10g/Kg of soil. Rice susceptible cultivar Taichung native 1 (TN1) was taken for *in-vivo* evaluation of all the isolated bioagents against *Xoo.* Potential *Trichoderma* isolate's spores and *Bacillus* isolate's culture (selected based on *in-vitro* studies) were harvested with the help of autoclaved water and the obtained suspension optical density (OD_{600nm}) was maintained for 1.0 and 0.4 respectively. The seeds were first soaked in water for 12 hours and then with all the bioagent's suspension for 12 hours. Twenty-five days after sowing, the seedlings were dipped in each bioagents solution containing 2% carboxymethyl cellulose (CMC) in separate beakers for 6 hours. These seedlings were then transplanted into pots of size 30x25cm maintaining 5kg of soil. After 30days of transplanting, the soil was again treated with bioagents @50g/ pot. Suspension of *Xoo* culture was prepared (OD_{600nm}=0.4) from freshly grown culture and was inoculated by clip-inoculation method on 40th day after transplanting (DAT). Disease screening was done at two intervals 14th day after inoculation (DAI) and 21st DAI. Data for plant growth promotion activities were recorded

upto maturity. From *in-vivo* results analysis, the potential bioagent isolates and pathogen were taken for scanning electron microscopy to know their detailed structure. The preparation of samples was done by following the procedure given by Bozzola and Russell (1999). The fresh samples were fixed in 2% glutaraldehyde prepared with 0.1M phosphate buffer (pH 7.2) for a day at 4°C. The samples were again subjected to 2% aqueous osmium tetroxide for 4h followed by dehydration with alcohol and EMS 850 CP dryer. Using automated sputter coater (Model- JEOL JFC-1600) the samples were mounted with double sided carbon conductivity tape and a thin layer of gold coat for 2 minutes. JEOL JSM-5600 SEM was used for observation of samples. The potential isolates of fungal and bacterial bioagents were taken for genomic DNA isolation, 18s rRNA (ITS region) and 16s rRNA sequence analysis, nucleotide sequencing and NCBI submission respectively. The freshly grown culture's cells (Log phase) of bacterial isolates in LB and fungal isolates in PDB were harvested and collected by centrifugation at 11,000 rpm for 10mins and taken for DNA isolation and purification using MN (Machery-Nagel) kit. The quantification of DNA was done using agarose gel electrophoresis (0.8%) as well as Nanodrop. For bacterial DNA, 16S rRNA region (~1500bp) was amplified by using universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3'). The PCR was performed by preparing master mixture with 50ng DNA template, 0.5 µM primers, 2.5mM dNTP mix, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 5 U *Taq* polymerase. Amplification was done by running 5min denaturation at 94°C followed by 30cycles of amplification with denaturation at 94°C for 30s, annealing at 60°C for 20s, extension at 74°C for 45s and final extension at 74°C for 7min. For fungal DNA, 18S rRNA gene (~1200bp) (partial sequence); internal transcribed spacer 1, 5.8S rRNA gene, and internal transcribed spacer 2 (complete sequence); and 28S rRNA gene (partial sequence) was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and LR3R (5'-GGTCCGTGTTTCAAGAC-3') primers. The amplification was done by running 30cycles of denaturation at 94°C for 1min, annealing at 50°C for 90sec followed by one more annealing at 72°C for 90sec, extension at 74°C for 45s and final extension at 74°C for 10min.

PCR product was analysed on 1% agarose gel containing ethidium bromide and visualised under UV transilluminator. Desired band from PCR product was purified using QIAGEN gel extraction kit. Sanger sequencing was done at Eurofins Genomics, India. NCBI BLAST system was used for nucleotide homology analysis for 16S and 18S region for bacteria and fungi respectively. For analysis of evolutionary pattern among closest lineages, phylogenetic tree was constructed by acquiring the sequence database of our isolates and homologous nucleotide sequences from NCBI, aligning them in one FASTA format file and designing the tree in MEGA software. On the basis of more than 90% similarity sequence with the tested sequences, database was collected and aligned for making phylogenetic tree using ClustalX version 2.0.11 and MEGA version 6.06. Studies at *in-vitro* level was performed twice with three replications. The experiment at glasshouse level was conducted for four replications in completely randomised design (CRD). One-way analysis of variance (ANOVA) having Post hoc test with Duncan's multiple range test (DMRT) at 5% ($P \leq 0.05$) significance level was performed in SPSS 20.0 software. Regression analysis and graphs designing were done in Microsoft Excel (2019).

Results and Discussion

From seven different places of four rice growing districts of Telangana *viz.,* Hyderabad, Khammam, Nalgonda and Suryapet, 10 isolates of *Trichoderma* and 5 isolates of *Bacillus* were isolated. The major soil type found was black cotton soil and red soil. The fungal isolates were mainly found in red soils with the pH range of 5.8-7.2 and the bacterial isolates in black cotton soil with a pH range of 7.4-8.3 (Figure 1). Telangana state is situated in upland region of Deccan (peninsular India) plateau which is covered with gneissic rock. As a result of erosion, the topography of the region consists of majorly red sandy soil and black soil in certain parts of the area. These soil type have different pH range which nurture various microbial diversity in soil (Mahesh *et al.,* 2018). On the basis of morphological and microscopic features, fungal and bacterial bioagents were identified (Table 2 and 3).

Table 2: Morphological characterisation of isolated fungal bioagents

Isolates	Radial growth (cm) in TSM after 4 days of incubation	Colony structure and colour of spores after 4 days of incubation	Spore maturation period	Chlamydospore	Conidiospores
T1	3.0±0.02	Thin mat of mycelium with dispersed dark green spores	5	Absent	Rarely branched
T2	3.1±0.01	Thick mat of mycelium with dispersed dull green spores	6	Absent	Broad and verticillate
T3	3.6±0.05	Thick mat of mycelium with dark green spores clustered in ring	4	Present	Branched, pyramidal structure
T4	2.0±0.01	Thick fluffy mat of mycelium light green spores	3	Present	Infrequently branched
T5	3.8±0.01	Thin mat of mycelium with light green spores	4	Absent	Divergently branched at right angle
T6	4.5±0.02	Thin mat of mycelium with clustered light green spores	2	Present	Branched, bottle shaped phiallids
T7	4.3±0.00	Thick mat of mycelium with dark green spores	2	Present	Long and infrequently branched, verticillate
T8	3.0±0.01	Thin mat of mycelium with yellowish green spores	5	Absent	Divergently branched at right angle
T9	3.4±0.05	Thin mat of mycelium with dull green tufts of spores	8	Absent	Clustered, verticillate
T10	3.9±0.03	Thin mat of mycelium with dispersed green spores	4	Absent	Divergently branched at right angle

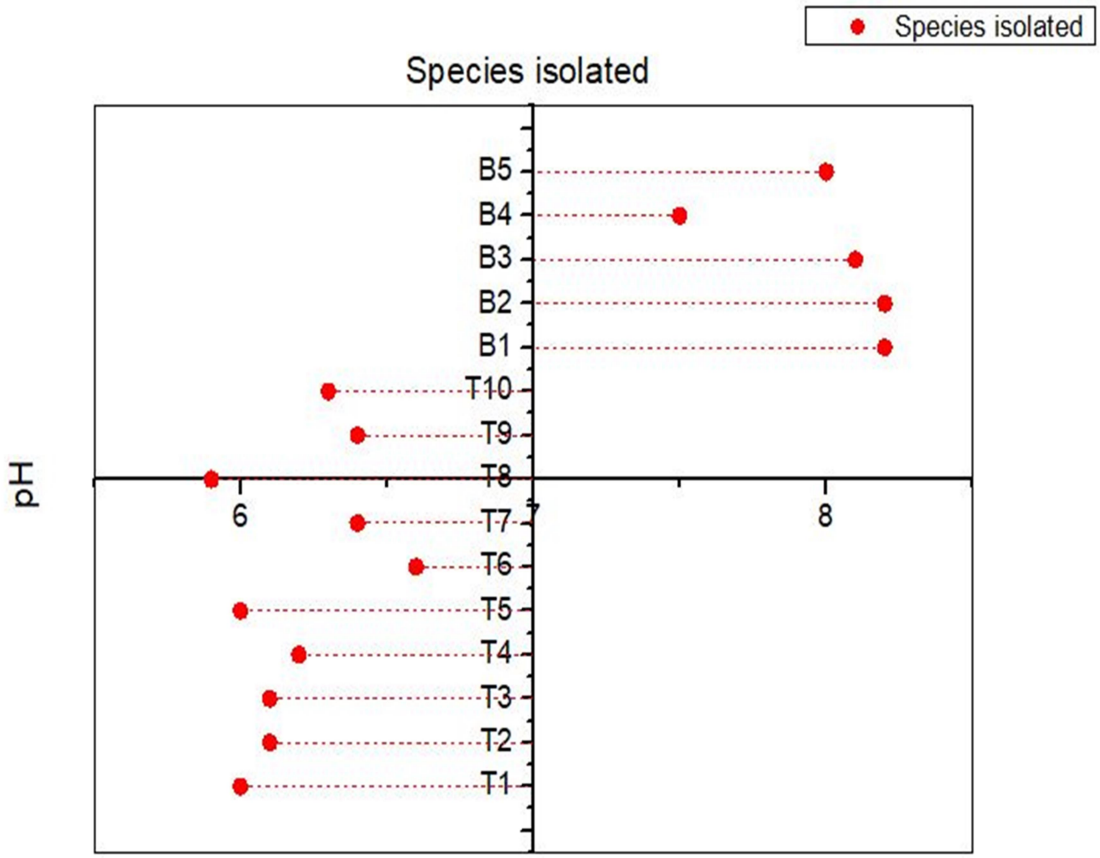
Note: Data depicts the mean of three replications

Table 3: Morphological characterisation of isolated bacterial bioagents

Isolates	Growth of culture (hours)	Colony configuration	Colour on nutrient agar	Shape of isolates
B1	18±0.50	Circular lobate with irregular margin and flat	White	Ellipsoidal
B2	36±0.38	Circular, slightly granular but not dry	Dull grey	Oval
B3	24±0.58	Flat or slightly convex, irregular	Off-white	Ellipsoidal
B4	30±0.65	Oval, opaque, dry	Off-white	Oval
B5	21±0.57	Round, smooth, moist	White	Rod

Note: Data depicts the mean of three replications

Figure 1: Distribution of species in the soil samples at different pH



Note: The soil samples collected from different regions of Telangana were tested for its pH content. X-axis represents the pH range and Y-axis represents different bioagents isolated.

Fungal bioagent identified as *Trichoderma atroviride* and bacterial bioagent *Bacillus subtilis* are generally cosmopolitan and free-living organism which are commonly found in soil. This nature makes them an ideal candidate in biocontrol programmes against a range of phytopathogenic fungi or bacteria in different environmental habitats (Reithner *et al.,* 2011; Ashwini and Srividya, 2014). They maintain symbiotic relationship with plants and act as potential decomposers. They colonise the root surface or lives endophytically and helps to increase plant growth. As decomposers, they make nutrients as well as play significant role in nitrogen and carbon cycle for plant development (Lieckfeldt *et al.,* 1999). Among the fungal bioagents, T6 isolate was showing highest production of IAA (69.73 mg/ ml), PS (153.16 µg/ml), siderophores (92.70 %siderophore units), chitinases (32.52 min^{-1} mg^{-1} Protein) and β-1,3-gucanases (1.93 nmol/s/ml) (Figure 2) (Supplementary Table 1). Among bacterial bioagents, B1 isolate was showing maximum production of IAA (10.97 mg/ ml), PS (65.87 µg/ml), siderophores (40.35 % siderophore units) and biofilm (1.48 OD_{600nm}) (Figure 3) (Supplementary Table 2). Plant growth promoting and antagonistic activities of the bioagents on rice plants has been attributed with the production of indole acetic acid, phosphate solubilisation, siderophore, chitinase, β-1,3-gucanases and biofilm. The phytohormone IAA helps in the enhancement of root and shoot length of the plant (Naziya *et al.,* 2020; Chinnaswami *et al.,* 2021). The ability to hydrolyse organic and inorganic insoluble phosphorus compounds to soluble P helps plants to take up phosphorus easily and utilise for further developmental processes (Prakash and

Table 4: Evaluation of antagonistic activity of fungal bioagents against *Xoo.*

Isolate	Radial growth of *Xoo* (96 hrs of incubation)	Percent inhibition
T1	0.52±0.50	54
T2	0.76±0.33	36
T3	0.61±0.50	45
T4	0.49±0.96	63
T5	0.51±0.20	54
T6	**0.20**±0.58	**81**
T7	**0.35**±0.65	**72**
T8	0.51±0.30	54
T9	0.72±0.43	36
T10	0.58±0.33	54
Control	1.10±0.74	-

Note: Data represents the mean of three replications with standard error values

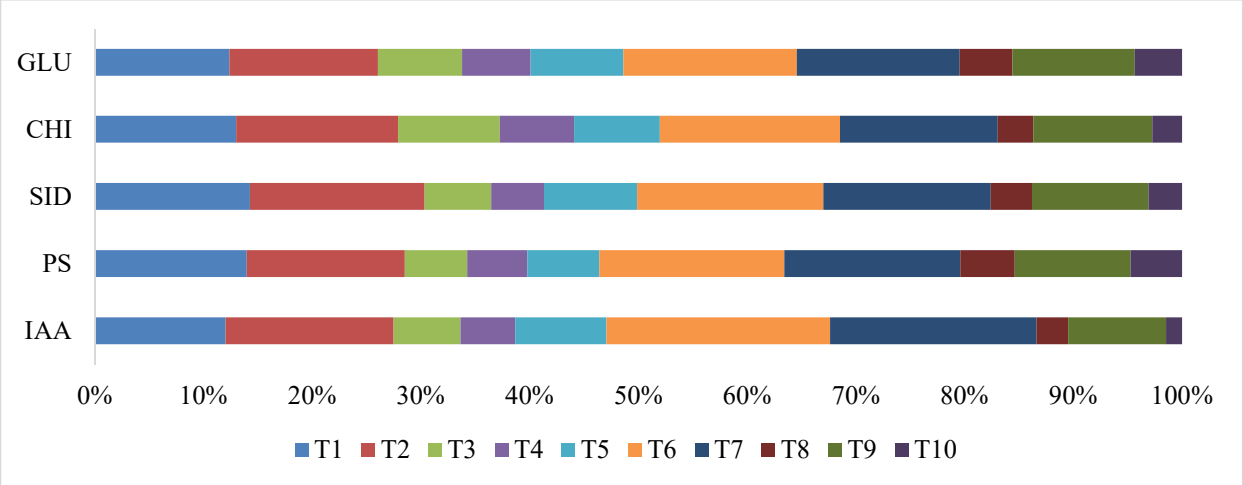
Table 5: Evaluation of antagonistic activity of bacterial bioagents against *Xoo*

Isolates	Zone of inhibition (cm)
B1	2.46±0.31
B2	1.33±0.50
B3	1.80±0.24
B4	1.59±0.82
B5	2.01±0.12
Control	-

Note: Data represents the mean of three replications with standard error values

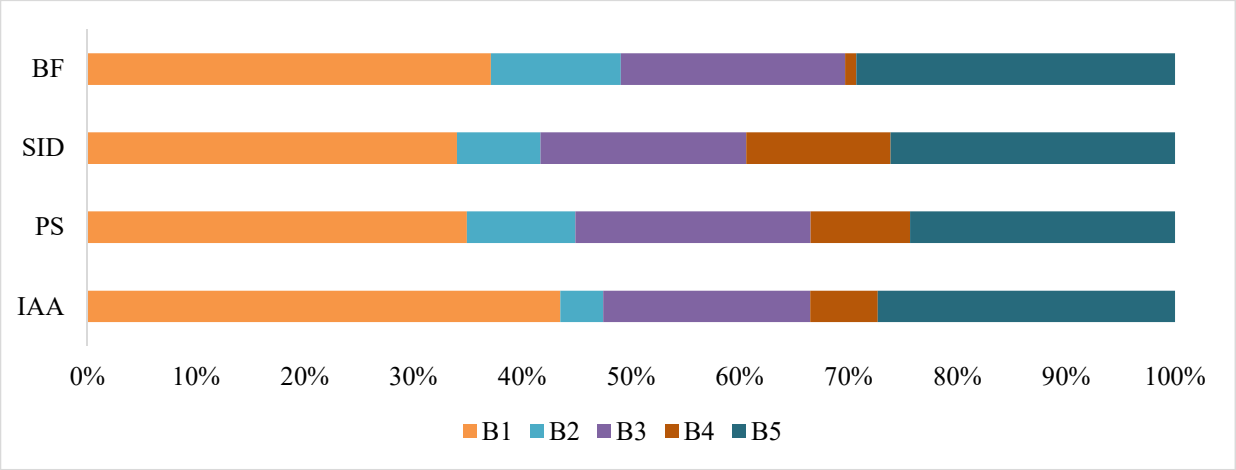
Arora, 2019). The high affinity system to uptake iron from the environment by plants with the help of these bioagents may enhance plant growth promoting activity (Scavino and Pedraza, 2013). The cell-wall degrading enzymes (chitinase and β-1,3-gucanases) and production of biofilm helps the plants to induce resistance and kill the pathogen (Naziya *et al.,* 2020). Typical pin head size, mucoid, convex yellow colonies were identified as *Xoo* and was streaked on MWA plates. Pathogenicity of the isolate was proved on rice susceptible cultivar: TN1 and the growth of pathogen was measured and compared on the basis of standard evaluation system (SES) scale. The per cent leaf area diseased was found to be more that 70% after 21days of *Xoo* inoculation. Based on reduction in the radial growth of *Xoo*, the per cent inhibition of pathogen by T6 (81%) was highest followed by T7 (72%) (Table 4). In case of *Bacillus* culture filtrate, each isolate was successfully able to reduce the growth of *Xoo.* The lethal concentration for each isolate was optimised to 60% (v/v). Clear zone of inhibition was found after 72 hrs of incubation and was highest in B1 (2.46 ± 0.31cm) when compared with other isolates (Table 5). Their antagonistic capacity has made their importance to be recognised in production of biopesticides, biofertilizers and phytostimulants (Kumar and Singh, 2015).

Figure 2: Stacked bar representation of different biochemical characters of *Trichoderma* isolates



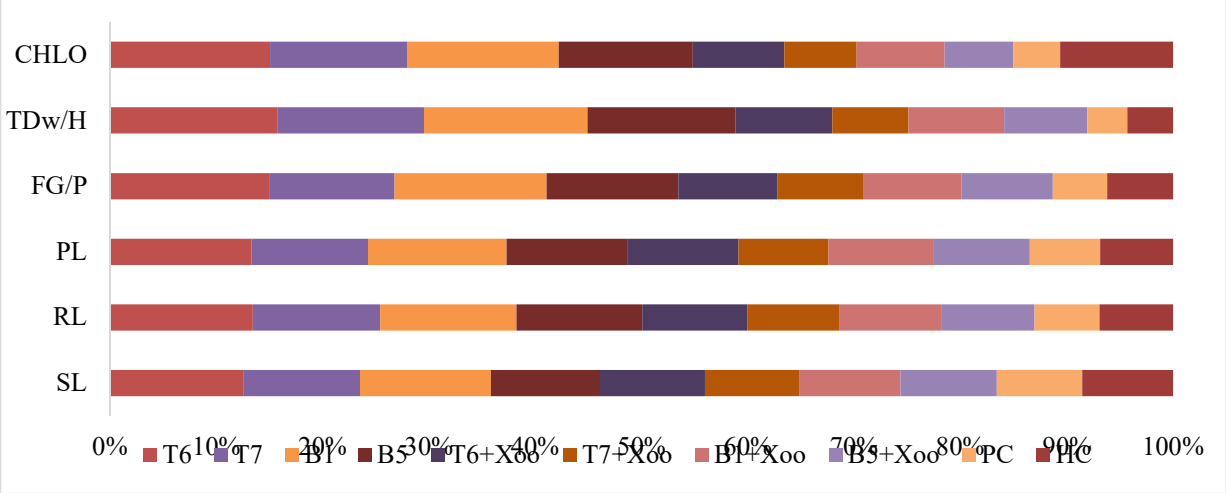
Note: Data depicts the mean of three replications. Stacked bar (100%) represents percentage of the whole of each group and are plotted by the percentage of each value of the total amount in each group. GLU: Glucanase, CHI: Chitinase, SID: Siderophore, PS: Phosphate solubilisation and IAA: Indole acetic acid

Figure 3: Stacked bar representation of different biochemical characters of *Bacillus* isolates.



Note: Data depicts the mean of three replications. Stacked bar (100%) represents percentage of the whole of each group and are plotted by the percentage of each value of the total amount in each group. BF: Biofilm, SID: Siderophore, PS: Phosphate solubilisation and IAA: Indole acetic acid

Figure 4: Stacked bar representation of morphological and yield parameters of rice plants in different treatments under glasshouse conditions.



Note: Data depicts the mean of three replications. Stacked bar (100%) represents percentage of the whole of each group and are plotted by the percentage of each value of the total amount in each group. CHLO: Chlorophyll, TDw/H: Total dry weight/hill, FG/P: Filled grains per panicle, PL: Panicle length, RL: Root length, SL: Shoot length, PC: Pathogen control and HC: Healthy control

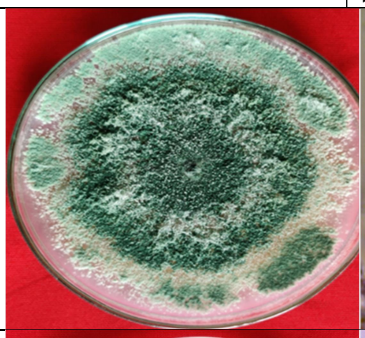
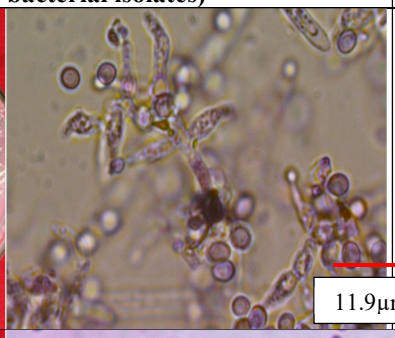
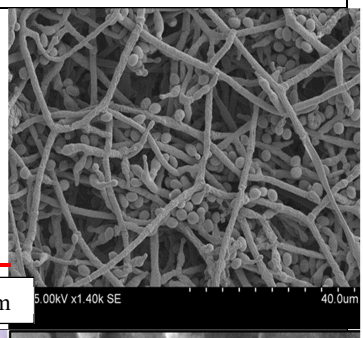
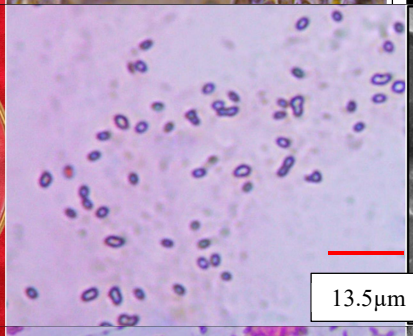
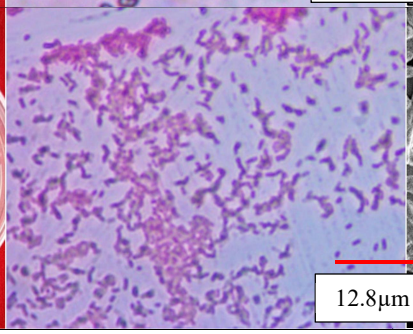
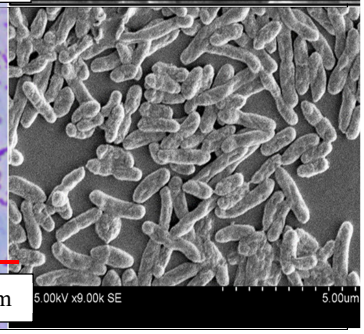
The *in-vitro* growth inhibition of *Xoo* by *Trichoderma* species was may be due to competition of nutrients which reduces the growth and biofilm production of *Xoo*. The saprophytic ability of *Trichoderma* sp. might be the reason for the overgrowth of bioagents on the pathogen (Mukherjee *et al.,* 2013). In case of *Bacillus* species, the growth of *Xoo* was inhibited due to release of antibiotics by bioagents (Antibiosis) (Lahlali *et al.,* 2013). From *in-vitro* experiment results, two potential *Trichoderma* (T6 and T7) and *Bacillus* (B1 and B5) isolates were taken for *in-vivo* study. In comparison with positive and negative control plants, rice plants treated with bioagents showed better growth promotion activities and reduced spread of *Xoo* in the infected leaves. Overall, the lesion length of plants treated with B1 isolate was minimum in comparison with T6, T7 and B5 (Table 6). However, enhancement in plant growth promoting attributes were highest in T6

Table 6: Evaluation of antagonistic activities of selected bioagents against *Xoo* under glasshouse conditions.

Treatments	Lesion length (cm)		Per cent diseased leaf area		Disease scoring	
	14th DAI	21st DAI	14th DAI	21st DAI	14th DAI	21st DAI
T6+*Xoo*	2.23±0.31^{b}	5.60±0.46^{bc}	1.06	11.63	3	5
T7+*Xoo*	2.87±0.15^{c}	6.37±0.61^{c}	1.30	15.87	3	5
B1+*Xoo*	2.13±0.15^{b}	4.90±0.26^{b}	0.94	7.82	2	4
B5+*Xoo*	2.37±0.25^{bc}	6.17±0.47^{c}	1.12	13.97	3	5
PC	4.83±0.31^{d}	11.73±0.50^{d}	1.58	35.86	4	7
HC	0.57±0.55^{a}	2.17±0.21^{a}	0.65	2.03	2	3

Note: Data depicts the mean of four replications. Numerical values with different letters are significantly different (P<0.05, DMRT, SPSS)

Table 7: Macroscopic and microscopic features of potential *Trichoderma* and *Bacillus* isolates along with *Xoo*

Organism	Colony morphology	Compound microscope pictures (40X for fungal and 100X for bacterial isolates)	SEM pictures
Trichoderma atroviride			
Bacillus subtilis			
Xanthomonas oryzae pv. *oryzae*			

followed by B1, T7 and B5 (Figure 4). The introduction of bioagents as seed or seedling or soil treatment increase the population of these microbes in soil and hence results in induced systemic resistance (ISR) when there is occurrence of pathogen (Mukherjee *et al.,* 2013). ISR helps in preconditioning of plant defenses by prior infection or treatment that results in resistance against

subsequent challenge by a pathogen (Choudhary *et al.,* 2007). The detailed size, morphology and surface of whole organism *viz.,* T6 (*Trichoderma atroviride*), B1 (*Bacillus subtilis*) and *Xanthomonas oryzae* pv. *oryzae* were analysed with the help of scanning electron microscopy at various magnifications (Table 7). The conidia of *Trichoderma atroviride* was smooth, round with size range from 2.5-3.9µm and the conidiophore was branched bearing bottle shaped phialides. The bacterium *Bacillus subtilis* was rod shaped, atrichous with a size of 1.45 x 0.62 µm and *Xoo* appeared as rod shaped with size of 1.2 x 0.52 µm. Based on results obtained from *in-vitro* and *in-vivo* studies isolate T6 and B1 were further proceeded for molecular identification. DNA of *Trichoderma* isolate T6 was amplified using ITS primers and *Bacillus* isolate B1 using universal primers specific to conserved 16S rRNA region and endoglucanase region (Figure 5). The amplified product was sequenced and obtained results were aligned against NCBI database. These sequences were compared with NCBI database and the percent similarity coefficient of T6 was 99% identical to *Trichoderma atroviride* and B1 was 97% identical to *Bacillus subtilis.* On the basis of percent similarity coefficient, 20 closest species of T6 and B1 database were analysed and phylogenetic tree was constructed to reconfirm the identity of bioagents. It was found that T6 was closely related to *T. atroviride* species and B1 to *Bacillus subtilis* (Figure 6 A and B). The culture sequence was submitted to NCBI (Figure 6 C). NCBI database percent similarity and phylogenetic tree analysis help us to know about the organism at species level. Phylogenetic tree construction helps to enrich our knowledge and understanding in finding out about which species the organism is related to (Soltis and Soltis, 2003). Comparisons of gene sequences of fungal and bacterial bioagents with the closest species database available at NCBI in a phylogenetic context has provided the most meaningful insights in identification and biology of these microbes.

Figure 5: Molecular Identification of selected native isolates *Bacillus* 1, *Trichoderma* 6 and *Xoo* using specific primers

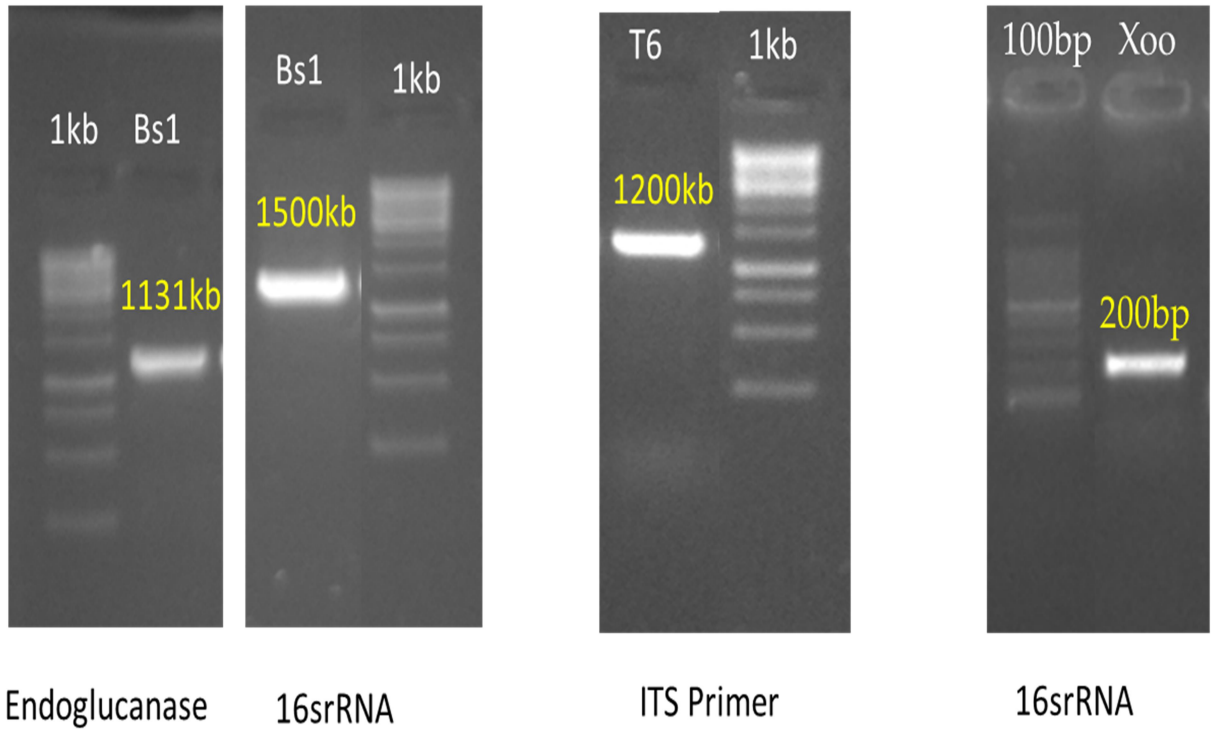
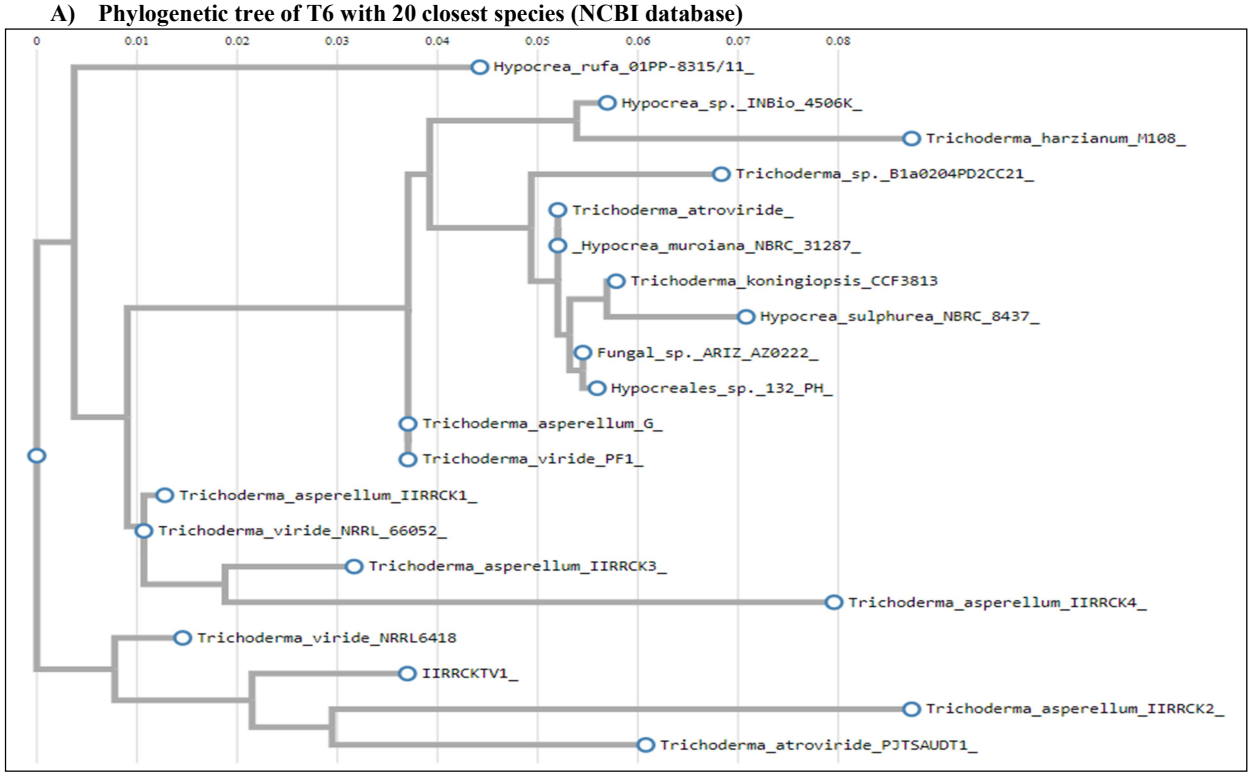
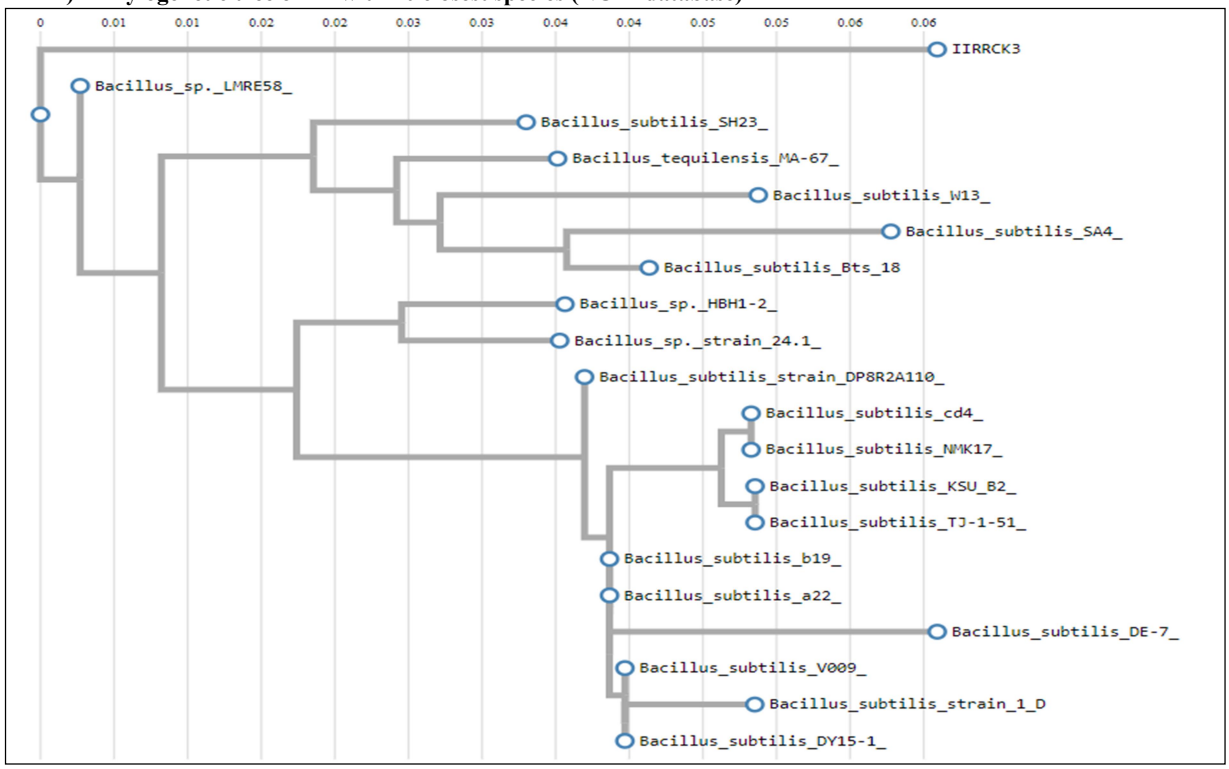


Figure 6: Identification of selected bioagents

A) Phylogenetic tree of T6 with 20 closest species (NCBI database)



B) Phylogenetic tree of B1 with 20 closest species (NCBI database)



C) Details of bioagents submitted to NCBI

Isolate code	Scientific name	NCBI accession number	Host	Collection date
T6	*Trichoderma atroviride*	MW188552	Rice rhizosphere soil	2019-08-23
B1	*Bacillus subtilis*	MT804606	Rice rhizosphere soil	2019-09-15

Conclusion

In the present study, two bioagents that induces rice plant growth and suppress its pathogen have been isolated and characterized. The application of bioagents on rice plants inoculated with *Xoo* has shown effective and successful reduction of the disease. The diverse soil distribution and rice cultivation in Telangana state have given an open chance to isolate beneficial microbes from rice rhizosphere soil, characterised their essential properties and utilize them accordingly against pathogens of rice. Molecular characterisation and phylogenetic analysis have helped to identify the organism up to species level. Work is in progress to develop an effective formulation for the controlled and safe release of the bioagents in the field conditions and can be used for the management of *Xoo*.

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