Chapter 2:

Isolation, screening and characterization of bacterial antagonists against *Xanthomonas oryzae* pv. oryzae BXO43

2.1. Introduction

Persistent and uncontrollable use of toxic chemicals attack untargeted organisms including the beneficial ones and cause other undesirable effects in the environment. Hence, making use of natural compounds showing antibacterial effect like biopesticides, can be considered as more rational and eco-friendly approach. Large group of bacteria inhabiting soil produce wide variety of structurally different antimicrobial metabolites. Isolating and studying such metabolites can fulfil the need of new biopesticides which could be used to protect the crops as well as environment.

Pseudomonas sp., *Bacillus subtilis, Lysobacter antibioticus* and other Plant Growth Promoting (PGP) strains have been reported to control bacterial leaf blight of rice caused by Xoo at field level (Udayashankar et al., 2011; Nagendran et al., 2013; Ji et al., 2008; Velusamy et al., 2006; Gnanamanickam et al., 1999). Antibiotics produced by fungus *Phomopsis longicolla* S1B4 (Lim et al., 2010) and Sphaeropsidins and its various derivatives have been shown by *in vitro* assay to be antibacterial against Xoo (Evidente et al., 2011). There is perpetual search for new antimicrobial agents and using antibiotics as crude extract produced by bacteria antagonistic to Xoo opens an opportunity for usage of wide varieties of antimicrobial agents. A potent antibiotic used in combination with other strategies could give a higher level of crop protection and improved rice yield.

Owing to long cultivation history and also because it is grown in diverse climatic, biotic and edaphic environments of diverse geographical regions; rice shows a remarkable microbial diversity (Lu, 1980). Hence, several components of the rice agro-ecosystem have been found to be rich sources of antagonists against pathogens of rice (Mew et al., 2004).

Taking this into account, source of isolation chosen were rice plants from different fields as well as other related plants of *Poaceae* family grown near rice field were chosen for isolation of bacterial antagonists against Xoo. The screening and selection of antagonists was carried out *in vitro* by agar plate method. Further, the characterization of selected isolates for their effect on growth of rice plant, their endophytic trait and the efficiency in controlling the disease symptoms of lesion formation on rice leaves was carried out. The best isolate as the antagonist for Xoo

pathogen was selected as the one giving highest protection against the lesion formation on rice leaves.

2.2. Materials & Methods

2.2.1. Bacterial pathogen and rice plant cultivar used

Bacterial pathogen: *Xanthomonas oryzae* pv. oryzae BXO43, an Indian pathovar was used for screening the bacterial antagonists in this study. Xoo BXO43 is a Rifampicin resistant strain of BXO1, which was isolated from a rice cultivar from Chinsuria, West Bengal in 1992. Xoo BXO1 belongs to pathotype III group which is widely distributed in India (Yashitola et al. 1997). Hence, Xoo BXO43 has been used in all the experiments as the test organism.

Rice plant: A susceptible variety of rice Native Taichung -1 (TN-1) have been used as host plant for infection studies of the phytopathogen. The rice variety was gifted by Dr. Pun, officer in-charge, Regional Rainfed Lowland Rice Research Station, Gerua, Hajo, Assam.

Plant sources: Various healthy rice plants and grass varieties were collected from fields of Main Rice Research Station, Anand Agricultural University Navagam, Gujarat and from the fields of University of Agricultural Sciences, Bangalore at Gandhi Krishi Vigyan Kendra (GKVK), natural rice field, Gokarna, Karnataka. sixteen Plants collected belonged to *Oryzae sativa* (rice), *Pennisetum purpureum* (napier grass), *Eleusine coracana* (finger millet), *Echinochloa colona* (jungle rice), *Eleusine indica* (indian goosegrass) and *Cenchrus ciliaris* (buffel grass) Sample collection was carried out in monsoon season and samples were stored in cold condition till further use. Bacterial antagonists were isolated from rhizospheric soil, phylosphere and root tissue and aerial parts (leaves and stem) of sample plants.

2.2.2. Media used for isolation and characterization of microorganisms

2.2.2.1. Luria Bertani (LB) broth: LB medium used for growth of bacterial isolates was composed of (g/l): casein enzymatic hydrolysate, 10.0; yeast extract, 5.0 and NaCl, 5.0. The medium was autoclaved at 121 ^{o}C/15 psi for 15 min.

LB agar was prepared by adding 1.5 % w/v agar in LB broth. LB agar plates were used for maintenance of different bacteria isolated in this study.

2.2.2.2. King's B Medium (KMB): KMB medium used for isolation of endophytes was composed of (g/l): Protease peptone, 20.0; K_2HPO_4, 2.5; $MgSO_4$, 6.0; Glycerol, 15 ml; agar, 15 and distilled water 1000 ml. The medium was autoclaved at 10 psi for 20 min.

2.2.2.3. Peptone-Sucrose (PS): PS medium as broth and agar used for growing pathogen Xoo BXO43 and for *in vitro* screening of antagonists using pathogen strain was composed of (g/l): Peptone 5.0 and Sucrose 20.0. PS agar was prepared by adding 1.5 % w/v agar in PS broth. The medium was autoclaved at 10 psi for 20 min.

2.2.2.4. Murashige and Skoog's nutrient medium (MS): MS medium used for growing rice plant was composed of : Sucrose 0.3 %, micro nutrients 76 mg/l (composition (mg/L): $MgSO_4.H_2O$, 16.90; H_3BO_3, 6.2; KI, 0.83; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $ZnSO_4.7H2O$, 8.60; $CuSO_4.5H_2O$, 0.025; $CoCl_3.6H2O$, 0.025; $FeSO_4.7H_2O$, 27.80; Na_2-EDTA, 37.30); macronutrients 4 gm/l (composition (mg/L): NH_4NO_3, 1650; $CaCl_2$; 332.2; $MgSO_4$, 180.69; NH_4NO_3, 1900; KH_2PO_4, 170) and Phytagel 0.2 %.The medium was autoclaved at 15 psi for 15 min.

2.2.3. Isolation of bacterial antagonists

Different plant parts like leaves, rhizosphere and roots were used for isolation of antagonists. Isolation of antagonists from rhizosphere and phyllosphere was carried out by pour plate method where test pathogen Xoo was seeded in the top agar and incubated for 6 hrs. Samples were rinsed under running tap water for 2 minutes and suspended in Phosphate-saline buffer (PSB) and vortexed thoroughly. Suspensions were serially diluted and dilution of 10^3, 10^4 and 10^5 were spread on top agar with sterile spreader and incubated for 48 hrs at 30 ^oC. Pure culture of isolates showing zone of inhibition were obtained and again checked for antagonism against Xoo by spotting on Top agar plate seeded with Xoo.

For endophyte isolation, 2 cm long pieces of plant tissues, stem or leaves were surface sterilized by rinsing thoroughly twice with deionized water and 3 rounds 30 % ethanol for 1 min and finally 3 -4 times sterile water. Then the ends of

the tissue (approx. 0.5 cm) cut with sterile scissors were placed on King's B agar plate and were incubated at 30 ºC for 3 -4 days. Bacterial growth observed near the cut ends of the tissue which was picked, serially diluted and plated on King's B agar plate and isolates showing different morphology were screened for their antagonistic activity by spotting them on PSA plates seeded with Xoo BXO43.

2.2.4. Culture maintenance and storage

Bacterial isolates were maintained on LB agar slants. They were regularly sub-cultured for short-term storage. Stock cultures of all isolates were prepared in 20 % glycerol and stored at -80 °C for long-term maintenance.

2.2.5. *In vitro* screening of antagonists of Xoo BXO43

Dual plate assay was carried out for selecting efficient bacterial antagonists. (Velusamy et al., 2006). Xoo was cultured in a 250 ml conical flask containing 100 ml of PS broth at 30 °C for 36 hrs on a shaker at 120 rpm. For screening of antagonists, 100 µl of 1.0 OD (10^{18} CFU/ml) Xoo BXO43 strain grown in PS broth was spread on PS agar plates. Isolated pure antagonist bacterial strains were grown in LB broth at 30 ºC for 16 hrs. the inoculum of the antagonistic bacteria was 5 µl of 1.0 OD_{600} cultures were taken and spotted individually on PSA plates and incubated at 30 ºC for 48 hrs. The inhibition of Xoo BXO43 by various bacteria was quantitated by zone of clearance/ colony size (Cz/Cs) ratio.

2.2.6. Physiological and Biochemical characterization of isolates

Gram staining and endospore staining was carried out for the isolates. Biochemical characterization of the bacterial isolates according to Bergey's manual of determinative bacteriology was carried out (Holt et al., 1994) as listed in the Table 2.4.

2.2.7. 16S rRNA gene sequencing and sequence analysis

The genotypic identification of the antagonistic bacteria isolates was done

using 16S rRNA gene sequence. Pure cultures were grown overnight in LB broth at 30 ^{o}C. Genomic DNA isolation of the isolates was carried out according to the protocol of Sambrook & Russel (2001). Amplification of 16S rRNA genes was done using ribosomal DNA universal bacterial primer set: 27F, 1107R and 1541R (Hoefel et al., 2005) (Table 2.1). PCR mixture of 50 µl contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 100 ng primer, 2.5 U of Taq polymerase (Invitrogen) and 10 -50 ng of genomic DNA template. Negative control contained all the components of PCR mixture except the DNA template. PCR amplification was carried out in a thermocycler (Applied Biosystem) using the following conditions: 1 cycle of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C; and 1 cycle of a final extension for 10 min at 72 °C. Amplified PCR products of size 1.5 kbps separated on a 1% agarose gel in 1× TAE buffer, containing ethidium bromide (1 µg/ml) were visualized and photographed using transmitted UV light at 295 nm. PCR bands were eluted from the gel by gel elution method according to Sambrook & Russel (2001). The amplicons were sequenced at AgriGenome Labs, India. The sequences were submitted at NCBI GenBank.

2.2.8. *gyrB* sequencing and ARDRA

The Gyrase subunit B (*gyrB*) gene was amplified from the genomic DNA of isolate S2 for its further characterization using degenerate universal primers , UP-1 and UP-2r (Table 2.1). Two type strains *B. altitudinis* 41KF2b^{T} and *B. pumilus* ATCC 7061^{T} were used as reference strains. 60 µl PCR mixture contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 100 ng primer, 2.5 U of Taq polymerase (Invitrogen) and 10 -50 ng of genomic DNA template. Negative control contained all the components of PCR mixture except the DNA template. PCR amplification was carried out in a thermocycler (Applied Biosystem) using the following conditions: 1 cycle of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 2 min at 72 °C; and 1 cycle of a final extension for 10 min at 72 °C. 1.3 kbps sized PCR products were visualized on a 1.5 % agarose gel in 1× TBE buffer, containing ethidium bromide (1 µg/ml) and visualized and photographed using transmitted UV light at 295 nm.

For ARDRA, 10 µl each of PCR amplicon of isolate S2 as well as type strains were used. The PCR amplicons were digested by restriction enzymes namely, Hha I, Msp I and Rsa I for 12 hrs at 37 ºC with 5 U of each restriction endonuclease. The restriction profiles were analysed and the fragment size were estimated using a 100 bp StepUp DNA ladder.

For sequencing, 50 µl of PCR product was loaded on a 1.5 % agarose gel and the band was eluted from the gel by gel elution method according to Sambrook & Russel (2001). The amplicons were sequenced at AgriGenome Labs, India using primers UP-1S and UP-2Sr and the sequence was submitted at NCBI GenBank.

Table 2.1: Primers used for the molecular characterization of the antagonistic isolates

Primer code	Sequence (5' – 3')	References
27 F	AGAGTTTGATCCTGGCTCAG	Hoefel et al., 2005
1107 R	GCTCGTTGTGGGACTTAACC	
1541 R	AAGGAGGTGATCCAGCCGCA	Yamamoto & Harayama, 1995
UP-1	GAAGTCATCATGACCGTTCTGCAYGCNGGN GGNAARTTYGA	
UP-2r	AGCAGGGTACGGATGTGCGAGCCRTCNACR TCNGCRTCNGTCAT	
UP-1S	GAAGTCATCATGACCGTTCTGCA	
UP-2Sr	AGCAGGGTACGGATGTGCGAGCC	

Y= C+T, N=A+T+G+C, R=A+G

2.2.9. Inoculation of rice plant with antagonistic bacteria

Rice cultivar Taichung Native-1 (TN-1) was used as it is a susceptible variety of rice for Xoo BXO43 strain. Seeds of the TN-1 rice variety were washed 3 -4 times thoroughly with sterile distilled water and were treated with 0.1 % $HgCl_2$ for 3 min followed by 2 min treatment with 70 % ethanol accompanied by continuous vigorous shaking. To remove traces of $HgCl_{2,}$ the seeds were washed with sterile distilled water three times and transferred to sterile 0.8 % water agar Petri-plates and incubated in dark for 3 days at 30 ºC. Sterile distilled water was sprinkled over the seeds after every 24 hrs to maintain humidity for proper germination. Bacterial isolates were grown in LB broth overnight in shaking condition at 30 ºC. Cultures in mid log phase (optical density of 0.4 -0.5 at 600 nm)

were centrifuged at 10,000 rpm for 10 min and the pellets were washed and suspended in saline and were adjusted to 0.2 OD_{600} to give 10^6-10^8 CFU/ml in saline. Germinated seeds were incubated with the isolates in shaking condition for 3 hrs. Treated seeds were transferred in sterile conditions in glass tubes having Murashige-Skoog (MS) medium and incubated in green house at 30 ^oC for 7 days. Effect of isolates on rice plant was measured as root and shoot length of the grown rice plant. Seedlings treated with saline only were taken as control to compare the effect of the bacterial isolates on growth of the plant. The experiment was carried out twice with three replicates each.

2.2.10. Detached leaf assay

Xoo BXO43 culture was grown in PS broth, washed and suspended in saline to give10^8 cells/ml. Selected bacterial antagonists were grown in LB in their log phase and 0.2 OD ($10^{9\text{-}10}$) were maintained as saline suspension for the assay. Leaves of 50 days old TN-1 rice plant were surface sterilized, kept in sterile Petri dish containing 0.8 % water agar and then the selected bacterial antagonists were individually inoculated by infiltration using pipette as 10^9-10^{10} cells per square centimetre of leaf area. After an interval of 3 hrs, 10^8 cells of Xoo BXO43 per square centimetre of leaf area were inoculated by infilteration using pipette at the cut ends and margins of the leaves. Lesions on leaves produced after the incubation of 5 days at 30 ^oC were scored. The leaves were photographed by Canon PowerShot SX400IS camera and for scoring the images of the lesions were then analyzed using ImageJ analyzer Fiji software (source: https://imagej.net/Fiji). Entire area of leaf as well as the area under the lesions were calculated with the help of this software. Further percent Diseased Leaf area (% DLA) was calculated as:

% DLA = (Area covered by lesions/ whole leaf area) x 100

Un-inoculated leaves and Xoo infected leaves were used as negative and positive control, respectively (Park et al., 2011; Burdman et al., 2004).

2.2.11. Efficacy of cell free culture supernatant of isolate S2 on preventing disease symptoms on rice leaves

The bioactive compound from S2 isolate was separated from the zone of

clearance shown by S2 isolate against Xoo BXO43 on agar plate assay. The agar pieces were homogenized in methanol by homogenizer (Remi) and centrifuged at 10,000 rpm for 10 min to separate agar. Methanol was separated and evaporated, and the residue was dissolved in water and filter sterilized using 0.2 micron filter (nylon filter, PAL). The effect of antibiotic on disease symptoms of Xoo was studied using detached leaf assay mentioned in above section 2.2.10. assay was similar, with a change in protocol of using filtrate instead of the bacterial culture followed by infilteration of the pathogen.

2.2.12. Antimicrobial activity of *B. altitudinis* S2

Different Gram negative and Gram positive bacteria namely *Escherichia coli, Salmonella typhi, Salmonella paratyphi* A, *Salmonella paratyphi* B, *Klebsiella pneumoniae* (MTCC 39), *Shigella dysenteriae*, *Staphylococcus aureus* were tested for their sensitivity to *B. altitudinis* S2. Dual culture agar plate was prepared where the test organism was plated on PS agar plate and *B. altitudinis* S2 was spotted at the centre of the plate. The plates were incubated at 30 ^{o}C for 24 hrs and sensitivity of the organisms was assessed in terms if zone of inhibition around the *B. altitudinis* S2. Similarly, few fungi namely *Fusarium oxysporum, Rhizoctonia bataticola*, *F. roseum, Magnaportha oryzae*. were also tested wherein a piece of fungus to be tested, was kept in the centre of the Potato Dextrose agar (PDA) plate and after 24 hrs freshly grown *B. altitudinis* S2 was spotted at one end of the plate. The plates were incubated at 30 ^{o}C for 3 -4 days and retardation of growth of the fungus towards the bacteria was observed.

2.2.13. Statistical analysis

Statistical significance of effect of bacterial antagonists on growth of rice plant and effect of isolates/extract on controlling disease by detached leaf assay was analyzed by one-way analysis of variance (ANOVA) using software Graphpad Prism (version 6.0). Differences among means were compared by Bonferroni's multiple comparisons test. $p < 0.05$ was considered as significant.

2.3. Results

2.3.1. Isolation of bacterial antagonist to Xoo

The isolates belonging to the natural bacterial flora from healthy plants of *Oryzae sativa* (rice) from geographically different places were chosen for obtaining the antagonists. Other plants from *Poaceae* family like *Pennisetum purpureum* (napier grass), *Eleusine coracana* (finger millet), *Echinochloa colona* (jungle rice), *Eleusine indica* (indian goosegrass) and *Cenchrus ciliaris* (buffel grass) grown near the rice field were also chosen as a source of bacterial antagonists against the phytopathogen Xoo BXO43. Isolation of antagonistic bacteria was done from rhizosphere, phylloplane and from root tissue as well as aerial parts of stem, leaves of the collected plant samples. Since, Xoo is a foliar pathogen aerial parts of plants were also used as sample for isolation of the antagonists against Xoo.

Isolation of bacterial antagonists from rhizosphere and phylloplane were isolated as mentioned in materials and methods, and were the purified bacterial cultures were further studied for their antagonism towards Xoo by agar plate method. Total 44 isolates obtained from the outer surface of the plant, showing different colony morphology were isolated, out of which 10 showed inhibition zone of Xoo BXO43 in *in vitro* assay. None of the isolates from phyllosphere showed antagonism. Surface sterilised root, leaves, stem were to obtain endophytes as mentioned in materials and methods. Fluid containing endophytic bacteria oozing out from the cut ends of the plant tissues, isolated in King's B medium for screening antagonistic bacterial endophytes, provided 46 isolates with different colony morphology of which 12 isolates showed antagonistic effect when tested with Xoo BXO43 in *in vitro* plate assay (Table 2.2).

Total 90 bacterial isolates were grouped in three groups based on the zone of inhibition produced by the isolates when tested against the test pathogen on agar plate. 68 isolates did not give any zone of clearance hence no antagonism, 8 isolates showed small zone around the colony and were grouped as moderate inhibitors, while 14 showing wide zones of clearance were categorized as strong inhibitors. Thus, strong inhibitors were selected for further characterization.

Table 2.2: Source of screened isolates and their inhibitory characteristics against Xoo BXO43

Plant species (no. of plants)	Location	Area/ Plant tissue	No. of isolates obtained	No. of Xoo antagonists
Rice plant (*Oryzae sativa*) (6)	Navagam, Gujarat and Bengaluru & Gokarna, Karnataka	Rhizosphere	12	5
		Root tissue	11	4
		Phyllosphere	10	-
		Leaf tissue	4	-
Finger millet (*Eleusine coracana*) (2)	Bengaluru, Karnataka	Rhizosphere	4	2
		Root tissue	6	2
		Phyllosphere	-	-
		Leaf tissue	1	-
Jungle rice (*Echinochloa colona*) (2)	Bengaluru, Karnataka	Rhizosphere	2	1
		Root tissue	2	-
		Phyllosphere	1	-
		Leaf tissue	1	-
Napeir grass (*Pennisetum purpureum*) (2)	Bengaluru, Karnataka	Rhizosphere	3	-
		Root tissue	3	2
		Phyllosphere	1	-
		Leaf tissue	4	2
Buffel-grass (*Cenchrus ciliaris*) (2)	Bengaluru, Karnataka	Rhizosphere	**4**	-
		Root tissue	3	-
		Phyllosphere	1	-
		Leaf tissue	3	-
Indian goosegrass (*Eleusine indica)* (2)	Navagam, Gujarat and Bengaluru, Karnataka	Rhizosphere	5	2
		Root tissue	6	2
		Phyllosphere	1	-
		Leaf tissue	2	-

Distribution of 90 bacterial isolates from different plant tissues of *Poaceae* family plants obtained from different geographical locations and isolates showing inhibition of Xoo BXO43

2.3.2. *In vitro* screening of antagonists of Xoo BXO43

Inhibition of isolates showing strong antagonism was further quantified by Clear zone (zone of inhibition) by Colony size (Cz/Cs) ratio. Finger millet isolate S2 obtained from root tissue showed the maximum ratio of 4.12 ± 0.13. Two isolates GL1 and S3 demonstrated spreading all over the plate inhibiting the growth of Xoo BXO43, as shown in (Fig. 2.1). Hence, their Cz/Cs could not be calculated. Based on Cz/Cs ratio, five isolates could be grouped in two group one showing Cz/Cs ratio above 3.5 and second, group of seven showing Cz/Cs ratio below 3.5.

First group included five isolates: L1, L21, R2, S2 and N4 (Table 2.3). Isolate S3 which showed effective inhibition of Xoo BXO43 was also selected for further studies along with above mentioned 5 isolates.

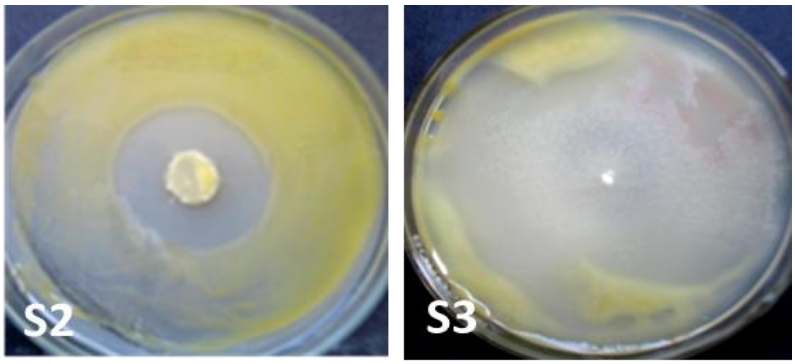


Fig.2 1: Inhition of Xoo BXO43 by the representative isolates
Plate assay showing inhibition of growth of Xoo BXO43 as a clear zone by a strong antagonistic isolate S2 and spreading growth by isolate S3

Table 2.3: Cz/Cs ratio of selected isolates against the pathogen Xoo BXO43 and the plant and tissue source from which the bacteria were isolated.

Isolate showing strong antagonism	Cz /Cs[a]	Plant source	Tissue or Area of isolation
R1	2.15 ± 0.31	Rice (Navagam)	Rhizosphere
R2	3.96 ± 0.07	Rice (Navagam)	Rhizosphere
L1	3.89 ± 0.54	Rice (Navagam)	Rhizosphere
L11	1.95 ± 0.35	Rice (Navagam)	Rhizosphere
L2	1.95 ± 0.35	Rice (Bengaluru)	Rhizosphere
L21	4.02 ± 0.33	Rice (Bengaluru)	Rhizosphere
S1	2.42 ± 0.13	Indian goosegrass	Rhizosphere
X1	2.90 ± 0.29	Indian goosegrass	Root tissue
S2	4.12 ± 0.13	Finger millet	Root tissue
S3	NA	Finger millet	Root tissue
B6	2.99 ± 0.38	Jungle rice	Rhizosphere
NR	1.93 ± 0.33	Rice (Navagam)	Rhizosphere
N4	3.91 ± 0.33	Napeir grass	Leaf tissue

GL1	NA	Rice (Gokarna)	Leaf tissue

[a] Cz /Cs of the twelve isolates tested using the pathogen Xoo BXO43 in *in vitro* agar plate assay. Data was obtained in three trials and depicts mean with standard deviation. NA: not applicable since the demarcating zone was not observed with these antagonists due to their spreading nature.

2.3.3. Characterization and identification of bacterial isolates antagonistic to Xoo BXO43

The phenotypic traits like morphological and biochemical characterization of the selected six isolates were carried out (Table 2.4). The isolates were identified based on genotypic traits consisting of 16S rRNA gene sequencing. Amplicons obtained were outsourced for sequencing and the sequence analysis by different online software programs like NCBI, RDB and Ezbiocloud provided the identification of the isolates. On the basis of BLAST analysis results of the sequences out of the six isolates sequenced, two were identified as *Bacillus subtilis*, while one each as *Bacillus altitudinis*, *Bacillus safensis*, *Pseudomonas* sp. and *Paenibacillus* sp. A dendogram based on the 16S rRNA gene sequences showing the closest match of the isolates was generated using software MEGA7 and is depicted in Fig. 2.2. The sequences were submitted in GenBank of NCBI with the accession numbers mentioned in Table 2.5.

2.3.4. Effect of isolates on growth of rice plant

In planta assay was carried out to examine if there are any adverse effects of the selected six isolates on the growth of the host rice plant. Rice cultivar TN1 was treated with six bacterial antagonists individually as described in section 2.2.9., and grown in green house condition for 7 days. The results quantitated in terms of length, wet and dry weight of root and shoot are depicted are depicted in Table 2.6. All isolates except isolate R2 did not affect the plant growth, while the isolate R2 caused a marked growth inhibition of rice plant (Fig. 2.3).

Table 2.4:Morphological and biochemical characterization of selected six antagonistic bacterial isolates

Isolates Characteristics	L1	L21	R2	N4	S3	S2	*B.altitudinis* 41KF2b^T	*B. pumilus* ATCC 7061
Gram character	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
Spore formation	+	+	+	-	+	+	+	+
Starch hydrolysis	+	+	+	-	-	-	-	-
Bile esculin test	+	+	+	+	-	-	-	-
Voges-Proskauer	-	-	+	+	-	-	-	-
Catalase test	+	+	+	-	-	+	+	+
Growth in 6.5% NaCl	-	+	+	-	-	+	+	+
Utilization of								
Sucrose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Manitol	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+
Fermentation of								
Sucrose	+	+	+	+	+	+	+	-
Glucose	-	-	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Indole production	ND	ND	ND	ND	ND	-	-	-
Lysine decarboxylase	ND	ND	ND	ND	ND	+	+	-
Phenylalanine deaminase	ND	ND	ND	ND	ND	+	+	-
Casein hydrolysis	ND	ND	ND	ND	ND	-	-	+
Gelatin hydrolysis	ND	ND	ND	ND	ND	-	-	+

+ indicates test was positive; - indicates test was negative; +ve is Gram positive and -ve is Gram negative; ND indicates not determined

Table 2.5: Isolates identified by 16S rRNA sequencing and their accession number in NCBI

Isolate	Identification	Accession number
L21	*Bacillus subtilis*	KU697355
L1	*Bacillus subtilis*	KU697356
S2	*Bacillus altitudinis*	KU697351
S3	*Paenibacillus* sp.	KU697352
N4	*Pseudomonas* sp.	KU697353
R2	*Bacillus safensis*	KU697354

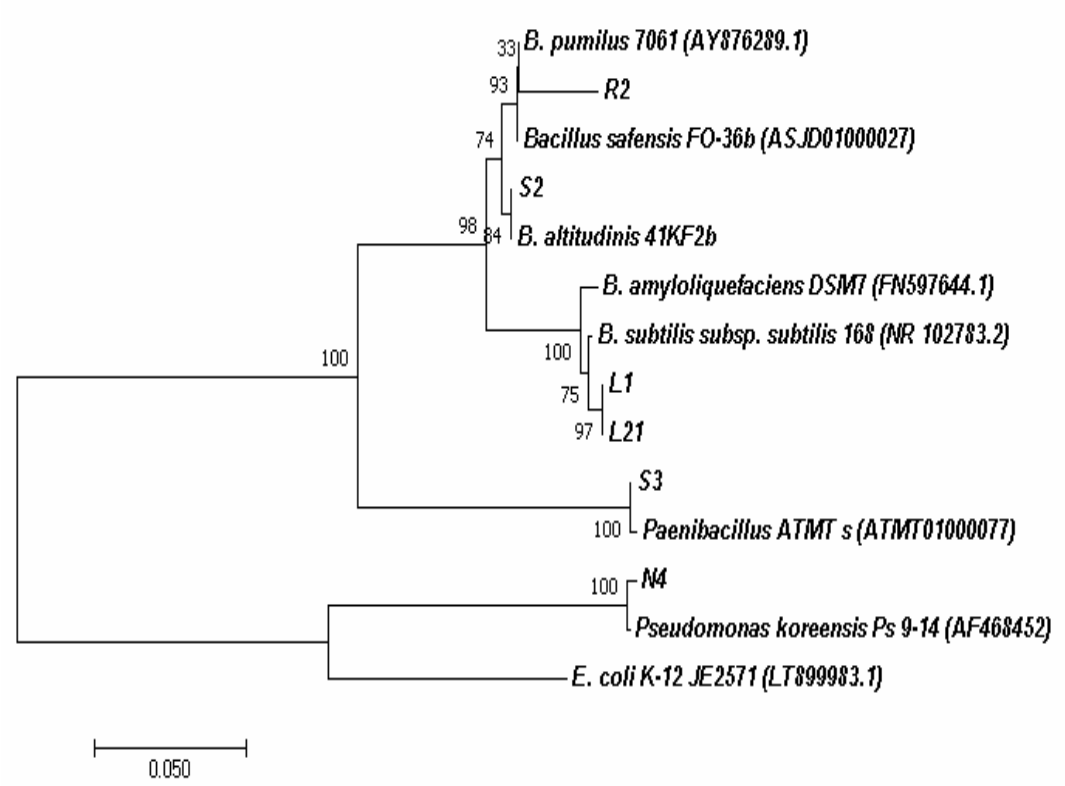


Fig.2 2: Phylogenetic tree based on 16S rRNA gene sequences of the six isolates and closely related type strains

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site using software MEGA7 (Kumar et al., 2016)**.** In the parenthesis are the NCBI GenBank accession numbers of the reference strains.

On the other hand as can be seen from the plant growth parameters of shoot and root length as well as wet and dry weight of shoot and root data of the isolates there is a slight increase in case of all the isolates as compared to control indicating a positive influence of the isolates on the rice plant. Since the isolate R2 was deleterious for the growth of the host rice plant it was not considered in the further studies.

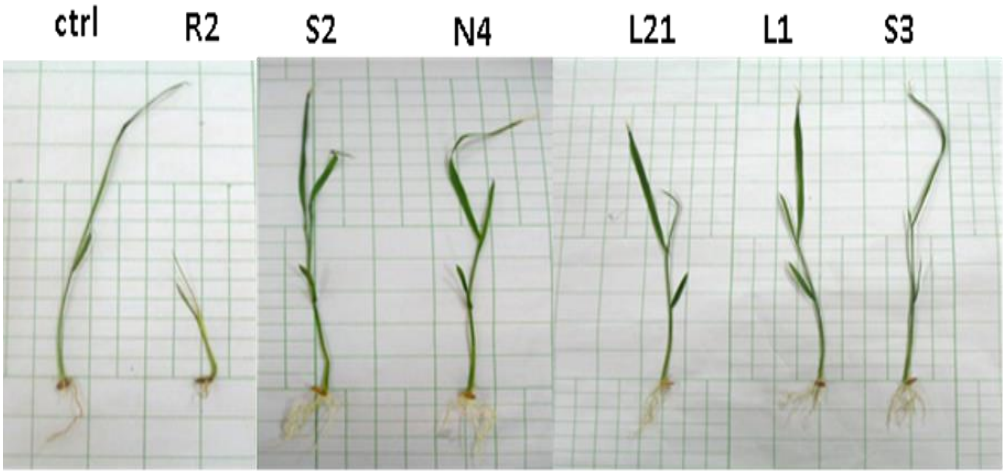


Fig.2 3: Effect of isolates on growth of rice plant
A representative experiment of rice plant grown for 7 days after the seedling were treated with the selected six isolates individually. 1- Control, 2 - *Bacillus safensis* R2, 3 - *Bacillus altitudinis* S2, 4 - *Pseudomonas* sp. N4, 5 - *Bacillus subtilis* L21, 6 - *Bacillus subtilis* L1, 7 - *Paenibacillus* sp. S3

Table 2.6: Quantitation of effect of isolates on rice plant in terms of root, shoot length and dry weight of root and shoot of rice plant.

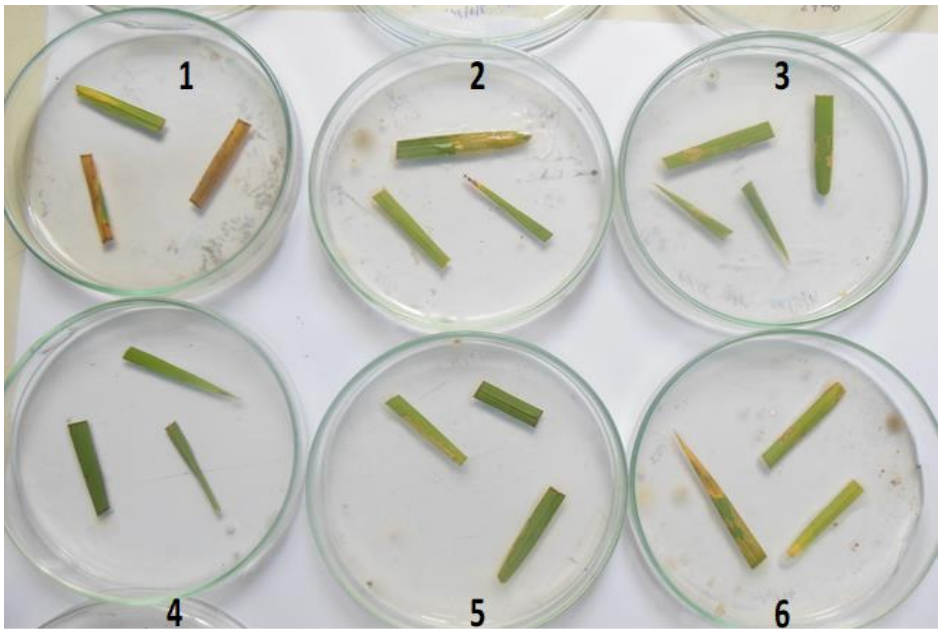
Isolate	Shoot Length (cm)	Root Length (cm)	Wet weight (mg)		Dry weight (mg)	
			Shoot	Root	Shoot	Root
Control	14.73 (6.93)	3.70 (1.30)	45.43 (27.97)	21.3 (6.91)	11.20 (4.47)	11.57 (0.51)
B. altitudinis S2	20.93 (1.55)	4.93 (0.12)	77.03 (12.59)	31.8 (2.50)	15.23 (2.21)	11.17 (0.74)
Pseudomonas sp. N4	21.07 (2.14)	5.17 (0.76)	69.97 (10.52)	45.33 (12.88)	14.70 (0.90)	14.70 (2.54)
Paenibacillus sp S3	26.00 (2.00)	4.03 (0.25)	68.80 (6.66)	38.23 (1.42)	50.80 (15.22)	11.77 (0.75)
B. subtilis L1	19.83 (0.76)	4.03 (0.70)	49.43 (5.71)	31.7 (10.15)	13.97 (0.72)	13.97 (0.93)
B. subtilis L21	19.93 (1.22)	5.17 (1.34)	46.93 (6.96)	25.1 (3.57)	15.97 (1.05)	11.53 (0.83)
B. safensis R2	6.00 (1.00)	1.20 (0.2)	15.60 (3.50)	11.3 (1.15)	8.10 (0.31)	4.50 (1.16)

Data represent mean and standard deviation of independent treatment of n=3 experiments. The values in parenthesis are indicative of the SD values.

2.3.5. Evaluation of inhibitory activities of the selected antagonistic bacteria on lesion development by detached leaf assay

The ability of the four isolates viz. *Bacillus subtilis* L21, *Bacillus altitudinis* S2, *Paenibacillus* sp. S3 and *Pseudomonas* sp. N4 to suppress the disease symptoms caused by Xoo BXO43 in rice leaves was examined by detached leaf assay. Among the two *Bacillus subtilis* strains the isolate L21 was selected as it gave better Cz/Cs. In the leaves treated with only Xoo, almost complete yellowing due to the disease symptoms was observed giving 70-100 % Diseased Leaf Area (% DLA) (Fig.2.4a (1)) and no lesions on leaves treated only with saline (Fig.2.4a (4)). Leaves treated with S3 isolate showed hypersensitivity response like symptoms (Fig. 2.4 a (3)). The adjoining Fig. 2.4 (b) shows the quantitation of reduction in rice blight disease symptoms in terms of % diseased leaf area (% DLA) brought about by each isolate as compared to the Xoo BXO43. The isolates S2 and S3 produced 13 and 20 % DLA, respectively, which is less as compared to isolates L21 and N4 18 and 30 % DLA, respectively.

(a)



(b)

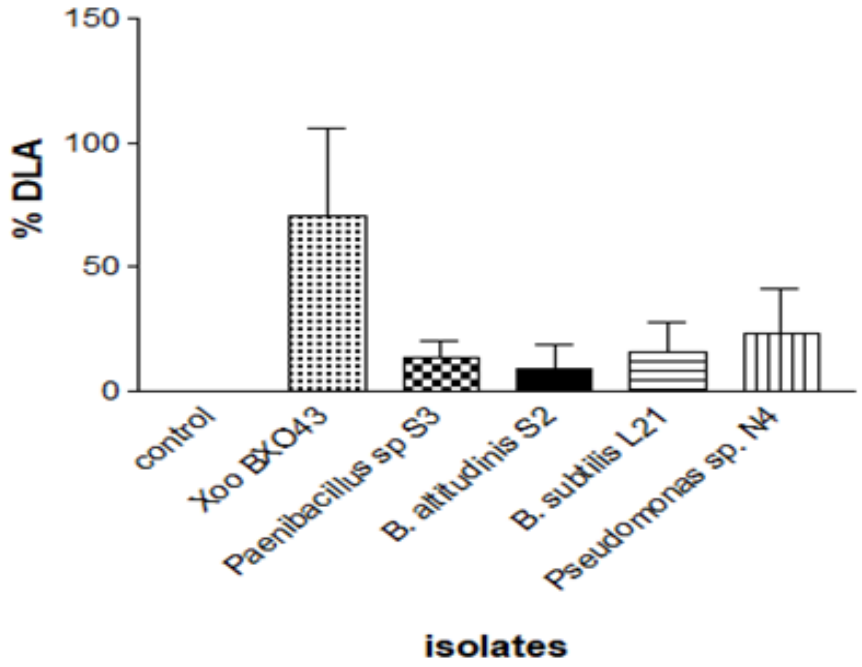


Fig.2 4: Effect of isolates on disease symptoms by detached leaf assay
(a) Rice leaves showing symptoms of disease as yellowish brown lesions when treated with Xoo BXO43 and no or less lesions were detected in leaves treated the selected antagonistic isolates prior to exposure to Xoo BXO43. (1 – Xoo BXO43; 2 – *Pseudomonas* sp. N4 and Xoo BXO43, 3 – *Paenibacillus* sp. S3 and Xoo BXO43; 4 – control; 5 – *Bacillus altitudinis* S2 and Xoo BXO43; 6 – *Bacillus subtilis* L21 and Xoo BXO43); (b) Results are mean of three independent experiments. Data was analyzed using one-way ANOVA measures followed by Bonferroni's multiple comparison test. For each. *, $p < 0.05$ level of significance.

2.3.6. Colonization studies of the selected antagonistic isolates using rice plant

To evaluate the ability of the isolated bacterial antagonists to colonize the root surface and to enter the internal tissue of the plant, root colonization studies were carried out using TN1 variety of rice plant. In the preliminary detached leaf assay the isolate L21 was poor in controlling the disease symptoms, hence it was eliminated from further studies. *Bacillus altitudinis* S2, *Pseudomonas* sp. N4 and *Paenibacillus* sp. S3 which were non-rice endophytic isolates isolated from root tissue and leaves were tested for their ability to colonize as an endophyte as well as on root surface. Rice plants were artificially inoculated with bacteria in sterile condition. After 24 hrs, 10^6, 10^8 and 10^7 CFU/ml of isolates S3, S2 and N4 respectively were found to colonize the root surface. On 15^{th} day post inoculation, CFU of the three isolates S3, S2 and N4 was found to increase to values 1.4 x 10^7,

5.3 x 10^7 and 2.8 x 10^7, respectively. However, by 30th day, it was found to decrease to 6.3 x 10^6 and 7.0 x 10^5 in case of isolates S3 and S2, respectively. Isolate N4 was not detectable at 30th day. As shown in the Fig. 2.5, bacteria were able to colonize the root surface of the plant; while very little or no bacteria were recovered from the root and shoot tissue after surface sterilization of the plant parts. Uninoculated plant treated with only saline were taken as control in all colonization studies. In the uninoculated rice plant, tiny faint yellowish bacterial colonies were always recovered from different tissues. This was also observed in the rice plants inoculated with the antagonistic isolates where these tiny colonies appeared along with the inoculated strains. The colony morphology and antibiotic resistance of the recovered bacteria were tested for the inference of the endophytic colonization of the bacteria under study. *Bacillus altitudinis* S2 was Bacitracin resistant and showed a colony morphology of circular rings in the 48 hrs old culture plate. *Pseudomonas* sp. N4 showed greenish shiny slimy colony and ampicillin resistance. While, *Paenibacillus* sp. S3 showed typical trait of spreading over the agar plate (Fig. 2.1) and showed resistance against Bacitracin.

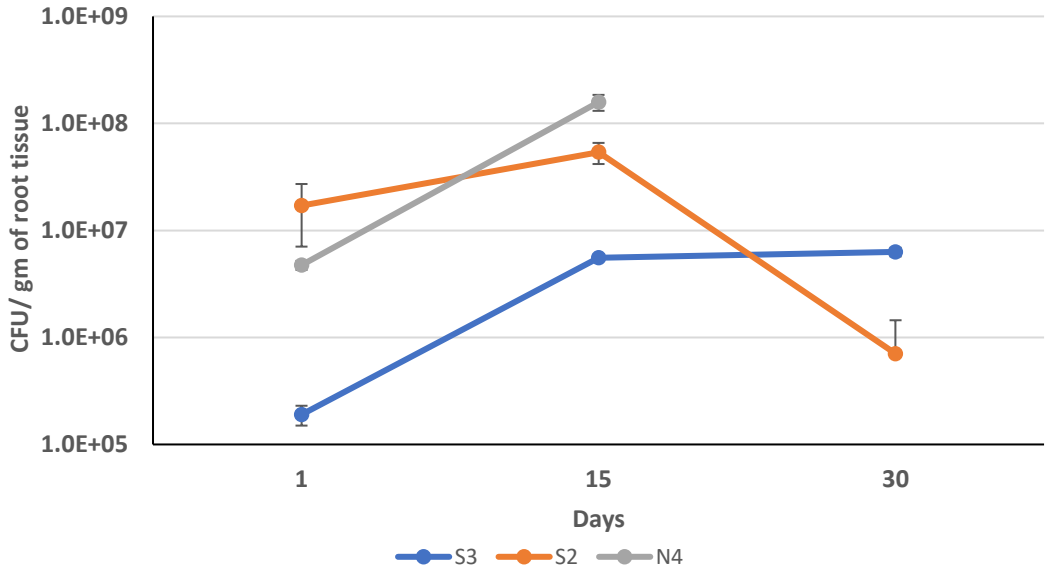


Fig.2 5: Recovery of the selected bacterial antagonists from root of the rice plants

The bacterial count were tested on 1st, 15th and 30th day after incubation. Isolate N4 was not detectable on 30th day. Experiments were done in three trials, each having triplicates. Each bar represents the mean of three experiments and its standard deviation.

2.3.7. Influence of cell free supernatant of *B. altitudinis* S2 on the disease symptoms of Xoo

B. altitudinis S2 demonstrated the highest Cz/Cs ratio for inhibition zone in plate bioassay against Xoo as test organism and effective inhibition of Xoo blight symptoms in the detached leaf assay. Also *B. altitudinis* S2 did not affect the growth of rice host. Therefore it was chosen for selected as strong antagonist of Xoo BXO43 and for further characterization on its mode of inhibition. For this, the cell free culture supernatant of *B. altitudinis* S2 was tested for its inhibitory effect if any, on the disease symptoms of bacterial blight caused by Xoo BXO43 using the detached leaf assay. The supernatant was recovered as described in section 2.2.11. As shown in the Fig. 2.6 (a), the leaves treated with only Xoo BXO43 showed yellowish brown lesions whereas the leaves treated with the cell free culture supernatant of *B. altitudinus* S2 prior to infection with Xoo BXO43 showed significant suppression of disease symptoms by reducing lesion formation to 14 ±1 % DLA as compared to 50 -60 % DLA obtained when leaves were infiltrated only with pathogen (Fig. 2.6 (b)).

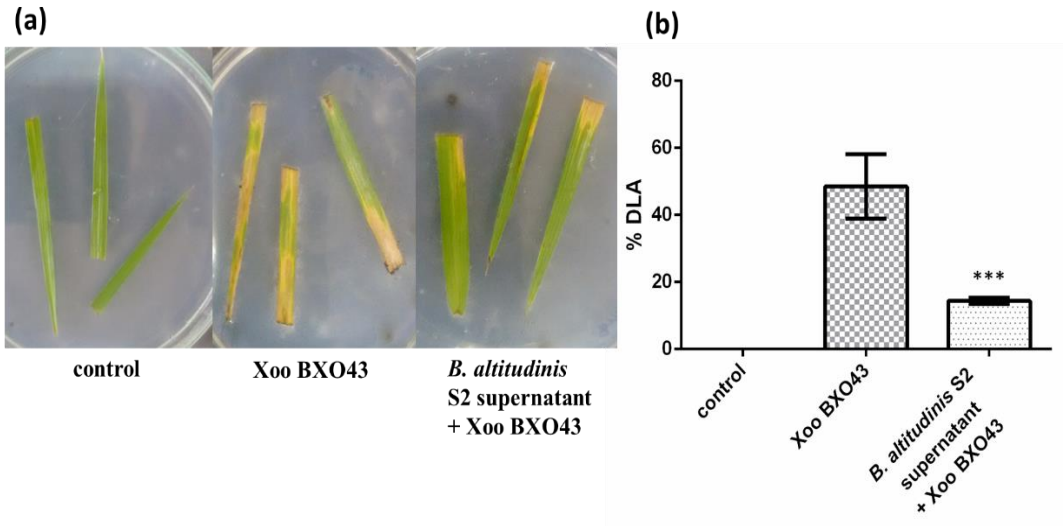


Fig.2 6: Effect of supernatant of *B. altitudinis* S2 in preventing the disease symptoms

(a) Detached leaf assay showing symptoms of disease as yellow lesions when infected with Xoo BXO43 and no lesions in leaves treated isolate S2. (b) Lesion formation quantified in terms of % diseased leaf area (% DLA). Mean and SD is of three trials. Statistical differences by one-way ANOVA followed by Bonferroni's multiple comparison test. Significance is shown by *** (p <0.001)

2.3.8. Confirmation of identification of *B. altitudinis* S2

For further characterization and elucidation of the mechanism of antagonism shown by selected isolate, it was important to confirm the species of *Bacillus* S2. On the basis of 16S rDNA sequencing, the online software programmes NCBI and RDB identified S2 strain as *Bacillus pumilus*, while the Ezbiocloud identified it as *Bacillus altitudinis*. As reported by Shivaji et al (2006), 16S rDNA characterization cannot distinguish amongst the closely related species of *Bacillus*. The isolate S2 belonged to *Pumilus* group in which both the *B. pumilus* and *B. altitudinis* are included. Hence, further characterization of the isolate S2 was carried out based on the distinguishing biochemical tests and another biomarker gene gyrase B (*gyrB*) sequence. The biochemical tests like gelatin liquification, citrate utilization, Phenyl alanine deaminase were distinct for two standard strains *Bacillus altitudinis* 41KF2b^T and *Bacillus pumilus* ATCC 7061(Table 2.4) (Shivaji et al., 2006). From biochemical tests, the isolate S2 showed matching characteristics as *Bacillus altitudinis* 41KF2b^T (Table 2.4). Further, *gyrB* gene sequence amplified using degenerate primers from isolate *Bacillus sp.* S2 was used to perform ARDRA and the ARDRA pattern of S2 *gyrB* gene was found to be closer to *B. altitudinis* (Fig. 2.7).

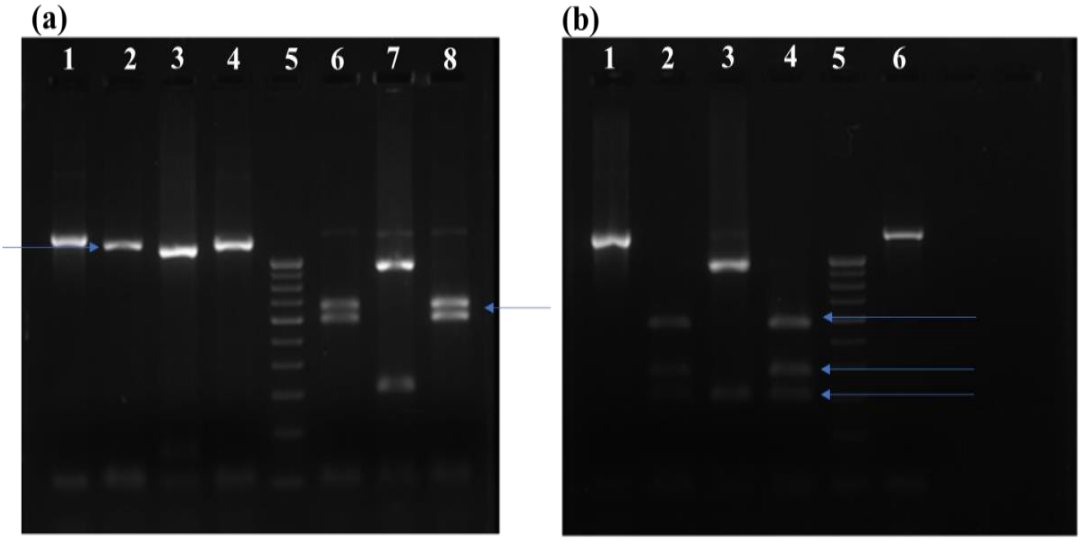


Fig.2 7: Gel image showing ARDRA band patterns of *gyrB* amplicon from isolate S2 and type strains *B. altitudinis* 41KF2b and *B. pumilus* ATCC 7061.
The amplicon size was 1.2 Kb.(a) single digest of restriction enzymes Hha I, Msp I and (Lane 1 – undigested *gyrB* amplicon from isolate S2, Lane 2 - HhaI digest of *gryB* (*B. altitudinis* 41KF2b), Lane 3 - Hha I digest of *gyrB* (*B. pumilus* ATCC 7061), Lane 4 - HhaI of *gyrB* (isolate S2), Lane 5 - 100 bp StepUp ladder, Lane 6 - MspI digest of *gyrB* (*B. altitudinis* 41KF2b), Lane 7 - MspI digest of *gyrB* (*B. pumilus* ATCC 7061), Lane 8 - MspI

digest of *gyrB* (isolate S2); (b) single digest of restriction enzyme Rsa I. (Lane 1 – undigested *gyrB* amplicon from *B. pumilus* ATCC 7061, Lane 2 - RsaI digest of *gryB* (*B. altitudinis* 41KF2b), Lane 3 - RsaI digest of *gyrB* (*B. pumilus* ATCC 7061), Lane 4 - RsaI of *gyrB* (isolate S2), Lane 5 - 100 bp StepUp ladder, Lane 6 – undigested *gyrB* amplicon from *B. altitudinis* 41KF2b

The PCR amplicon of *gyrB* was sequenced and these results were validated as can be seen in the phylogenetic dendogram generated based on *gyrB* gene sequence (Fig. 2.8). The sequence of *gyrB* gene was submitted in NCBI GenBank with accession number MH517594.

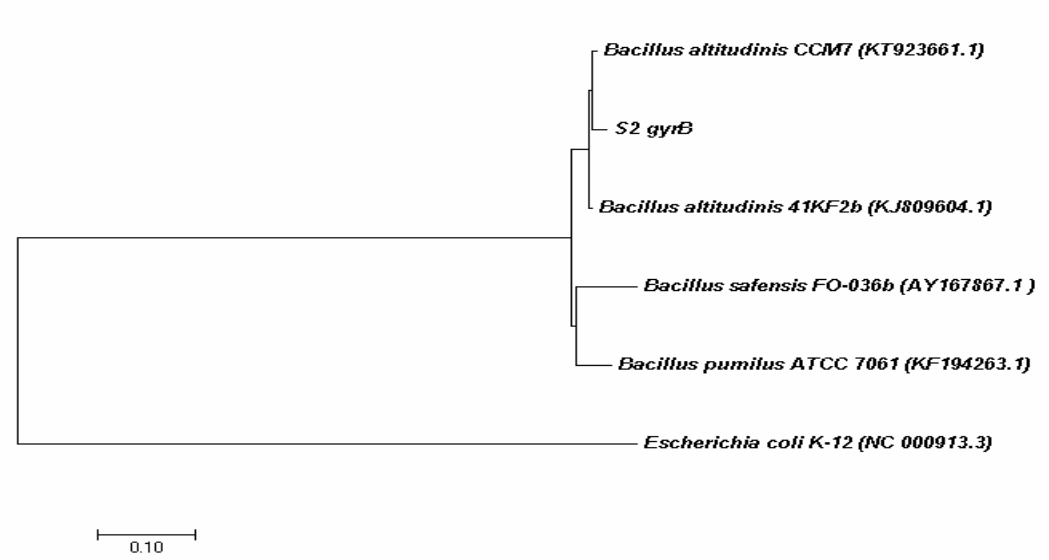


Fig.2 8: Phylogenetic tree based on *gyrB* gene sequence of isolate S2 and other related type strains of the *Bacillus* sp.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 1.33962754 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Evolutionary analyses was conducted in MEGA7 (Kumar et al., 2016)**.** In the parenthesis are the NCBI GenBank accession numbers.

2.3.9: Antimicrobial activity of *B. altitudinis* S2 on few bacterial and fungal strains

The antimicrobial activity of *B. altitudinis* S2 were tested against different bacteria *E. coli*, *Salmonella typhi*, *Salmonella paratyphi* a, *S. paratyphi* b, *Klebsiella pneumonia* (MTCC 39), *Shigella dysenteriae*, and *Staphylococcus aureus*. It did not exhibited any inhibitory activity against the common rhizosphere inhabiting soil bacteria like *Pseudomonas fluorescens* CHA0 and *Bacillus subtilis*.

Apart from Xoo, it is active against Gram positive bacteria *Staphylococcus aureus* ATCC 6538 P. Also, the isolate *B. altitudinis* S2 lacked any antifungal activity against the common fungal phytopathogens like *Fusarium oxysporum*, *Rizoctonia bataticola*, *F. roseum*, *Magnaportha oryzae*.

Table 2.7: Antimicrobial spectrum of *B. altitudinis* S2

Micro-organisms	Inhibition by *B. altitudinis* S2
Escherichia coli	-
Salmonella typhi	-
Salmonella paratyphi a	-
Salmonella paratyphi b	-
Klebsiella pneumonia (MTCC 39)	-
Shigella dysenteriae	-
Pseudomonas fluorescens CHA0	-
Bacillus subtilis	-
Staphylococcus aureus ATCC 6538 P	++
Fusarium oxysporum	-
Rizoctonia bataticola	-
Fusarium roseum	-
Magnaportha oryzae	-

-Indicates no inhibition of the organism, ++ indicates zone of inhibition of 15 mm observed.

2.4. Discussion

The aim of the research work undertaken here was to assess different eco-friendly agents; and to explore an integrated approach which could prevail effective for longer period of time in controlling the bacterial blight of rice which is caused by a Gram negative pathogen, *Xanthomonas oryzae* pv. oryzae (Xoo). Hence, the main objective of this chapter was the isolation of antagonistic bacteria to Xoo, as the microbe-based strategies are considered as an alternative to the conventional harmful chemical methods which cause serious threat to the environment. Bacteria of genus *Xanthomonas* show unique characteristics of host specificity (Chan & Goodwin,1999). For example, Xoo infects only *Oryzae sativa* (rice) and a few other related wild grasses (Ryan et al., 2011). Several potential antagonists from rice plant have been isolated and commercialized for disease management (Yang et al., 2008). Hence, different healthy plants from *Poaceae* family were particularly selected for isolation of antagonistic bacteria. Rice plants from different locations and other closely related non-rice plants like *Pennisetum purpureum* (Napier grass), *Eleusine coracana* (finger millet), *Eleusine indica* (Indian goosegrass), *Echinochloa colona* (jungle rice) were chosen for isolation of bacteria antagonistic to Xoo.

Studies have shown that beyond the rhizosphere, bacteria inhabiting other plant parts like internal tissue of root and leaves as well as phyllosphere produce different kind of secondary metabolites effective against different phytopathogens (Compant et al., 2016; Giddens et al., 2003; Lindow & Brandl, 2003). Therefore, bacteria from different areas associated with plants like root & leaf tissue, phyllosphere as well as rhizosphere were screened to isolate the antagonistic bacteria, using a virulent strain Xoo BXO43. From total 90 bacterial isolates, screening of antagonists was carried out in *in vitro* plate assay using high inoculum of pathogen (10^{17} CFU/ml). Fourteen isolates showed strong inhibition of Xoo BXO43 and were further subjected to secondary screening and categorized as per the Cz/Cs ratio in plate inhibition assay. Five isolates L1, L21, R2, S2 and N4 gave more than 3.5 Cz/Cs ratio and isolate S3 which did not show a zone but effectively inhibited the Xoo were taken for identification. Identification of these six isolates by 16S rDNA sequencing phylogenetically related them with *Bacillus, Paenibacillus* and *Pseudomonas* species. Many studies on biocontrol agents against various phytopathogens have shown to be contributed mainly by members of genera *Bacillus* and *Pseudomonas.* Also, these genera have been shown to be effective antagonists against Xoo (Sharma et al., 2017; Vasudevan, 2002; Gnanamanickam & Mew, 1992).

Before conducting further studies with the antagonists, it was important to study the effect of these bacteria on the growth of host rice plant. The effect of the isolates on the growth of the rice was further studied wherein all isolates except R2 which was identified as *Bacillus safensis* by 16S rRNA sequencing, had shown no harmful effect on the plant growth rather showed slight PGP effect on the growth of rice plant cultivar TN-1. Isolate R2 was eliminated from further studies due to its deleterious effect on growth of the host rice plant. Further, detached leaf assay was carried out to study the efficiency of the selected isolates to control the disease symptoms. Among the two *B. subtilis strains,* strain L21 was selected. Hence, four strains *Bacillus subtilis* L21, *Bacillus altitudinis* S2, *Paenibacillus* sp. S3 and *Pseudomonas* sp. N4 were tested for their ability to control disease symptoms on the rice by detached leaf assay. All the four isolates were able to reduce the disease symptoms on leaves. Amongst these, *Bacillus altitudinis* S2 was found to be most effective in controlling the disease symptoms on susceptible rice variety TN-1.

Xoo is an endophytic phytopathogen inhabiting the xylem vessels of the host plant. Hence, antagonistic bacteria competing for the ecological niche with the pathogen can be a good strategy to control the spread of the disease (Sturz et al., 2000). Old and Nicolson (1978) and Petersen et al. (1981) concluded that many bacteria from the rhizosphere are capable of entering the root tissue and as apoplastic pathway continue from root epidermis to shoot and many bacteria can move from root cortex into the xylem of the host plant. The bacteria producing bioactive metabolite can be used directly or the bioactive metabolite can be produced at large scale for their application as biocontrol agents for protecting the plants against the phytopathogens (Fravel, 2005). Both the methods, using bacteria or their bioactive metabolite as biocontrol agent have their own pros and cons. As the isolates S2 and S3 were isolated from root tissue and N4 from leaf tissue, the characterization of these strong antagonistic isolates as endophytes to the rice plant could give an effective method to control BB disease since Xoo have been reported to be the endophytic pathogen which inhabits the xylem vessels of the host plant. These isolates were found to be effective root colonizers; however, they were not found to behave as endophytes with the rice variety used in our study i.e. rice cultivar TN-1. Endophytic nature is a good attribute for biocontrol approach where bacteria are used to control the phytopathogen.

To understand the mode of inhibition of Xoo by *Bacillus altitudinis* S2, the cell free supernatant was examined for its efficiency to reduce or eliminate the lesion formation on rice leaves by detached leaf assay. The cell free supernatant was found to be effective in controlling the disease symptoms by detached leaf assay. This led to the conclusion that the antagonism of *B. altitudinus* S2 to Xoo is due to production of an extracellular metabolite. For identification of bioactive metabolite produced by the isolate and its further characterization, it is important to identify the bacteria at species level. Apart from 16S rRNA sequencing, another useful biomarker used was gyrase subunit B gene. ARDRA and further sequencing of the gene confirmed the identification of the isolate as *B. altitudinis* S2. Antimicrobial spectrum of the isolate *B. altitudinis* S2 exhibited inhibition of both, Gram positive and Gram negative bacteria but no antifungal activity. The results showed that amongst the tested organisms, apart from Xoo BXO43, *S. aureus* was

also sensitive. While other commonly found bacteria like *P. fluorescens* CHA0 and *B. subtilis* were not inhibited.