PCR-BASED IDENTIFICATION OF NATIVE *BACILLUS SUBTILIS* ISOLATES AND ITS *IN-VITRO* GROWTH SUPPRESSION ABILITY AGAINST *MAGNAPORTHE ORYZAE*

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ABSTRACT

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The present investigation focused on the molecular characterization of native rhizospheric *Bacillus subtilis* strains showing potential antagonistic activity against *Magnaporthe oryzae*. Morphological characters were the basis of identification for *Magnaporthe oryzae*. A total of 66 bacterial isolates were collected from rhizosphere soils of different agro-ecological zones of Bangladesh which were initially identified as *Bacillus* spp. based on their cultural and morphological characters. Dual culture technique was followed to screen potential antagonistic *Bacillus* isolates against *Magnaporthe oryzae*. Nine bacterial isolates showed growth suppression activity and the isolate BSL-17 showed the highest mycelial suppression against *Magnaporthe oryzae* by 85.12-87-30%. The isolate BSL-10 also showed a significant inhibitory (82.80%) effect against *Magnaporthe oryzae* next to BSL-17 followed by BSL- 31, BSL-41, BSL-27(ii), BSL-15(ii), BSL-21, BSL-26(iii) and BSL-8. Nine effective bacterial isolates were further confirmed as *Bacillus subtilis* by molecular analysis of 16S rDNA gene. Isolate, BSL - 17 showed 97% homology with *Bacillus subtilis* Bcx1 of Changchun city, China.

Key Words: *Bacillus subtilis*, Bio-control, *In-vitro* inhibition, *Magnaporthe oryzae*,16Sr DNA.

INTRODUCTION

Blast disease caused by *Pyricularia oryzae* Cavara [teleomorph: *Magnaporthe oryzae* (Hebert) Barr)], is one of the most destructive diseases of rice (*Oryza sativa* L.) in worldwide (Koutroubas *et al*. 2009). Rice blast pathogen is most common on leaves, causing leaf blast during the vegetative stage of growth, or on neck, nodes and panicle branches during the reproductive stage, causing neck blast (Bonman 1992). Neck blast is considered the most destructive phase of the disease and can occur without being preceded by severe leaf blast (Zhu *et al*. 2005). It had a wide host range infecting more than fifty plant species including rice, wheat, finger millet and barley (Talbot 2003). Every year it is estimated that rice blast destroy food more than enough to eat for 60 million people and 50% of the rice yield is lost in the field by the occurrence of blast (Barman and Chattoo 2005). In general, the disease causes 10-20% yield reduction in susceptible varieties, but in severe cases the loss may be up to 80% (Koutroubas *et al*. 2009). Over use of the chemicals has led to loss or decrease in their efficacy, toxicity to the environment, and harmful effects on the non-target organisms, which collectively affect their commercial applicability (Ongena and Jacques 2008). Moreover, continuous use of chemical may increase resistance of the pathogen to fungicides (Gossen *et al*. 2001; Mueller *et al*. 2002). Therefore, the greatest precautions should be concerned for the development of novel, durable and environmentally safe strategies to manage this devastating fungal disease.

The use of biological control in the management of agriculture pests and diseases is an effective alternative to the use of pesticides, which are often accumulated in plants and are lethal to beneficial organisms present in the soil. This is a more environmentally and friendly option than chemicals. Moreover bio-control might be effective against those pathogens which are difficult to control by conventional means. *Bacillus subtilis* is a bacterium which is nonpathogenic and lives in soil, often in association with roots of higher plants. *B. subtilis* cells are capable of forming dormant spores that are resistant to extreme conditions and thus can be easily formulated and stored (Piggot and Hilbert 2004). It also produces a var compounds with a broad spectrum of activities toward phytopathogens and that are able to induce host systemic resistance (Stein 2005; Butcher *et al*. 2007; Ongena and Jacques 2008).*B. subtilis, B.*

amyloliquefaciens and other closely related *Bacillus* spp. can control phytopathogen through producing various antimicrobial metabolites like surfectin, iturin and fengycin (Hou *et al*. 2006; Stein 2005).

Study and understanding of bio-control mechanism may bring benefit regarding the utilization of bio-control agents. Biological interaction might be involved in the mechanism of disease suppression which includes parasitism, competition, antibiotics and plant induced resistance. There is a need to develop beneficial and antagonistic microbes for making bio-fungicides around the glove. Multifarious ability of bio-control agents is suitable for crop production (Choudhary and Johri 2009). Very scanty research has been reported on the exploitation of *B. subtilis* for blast disease management in Bangladesh. Considering the above facts, this study was designed to isolate, identify and characterize *Bacillus subtilis* in order to develop effective strategy for sustainable management of rice blast disease caused by *Magnaporthe oryzae*.

MATERIALS AND METHODS

Isolation and Identification of *Magnaporthe oryzae*

The infected plant parts of rice (base of the infected panicle) were cut into small pieces and sterilized with 10% clorox solution for 30 seconds and then washed with distilled water for three times. The inocula were plated on moistened blotter paper and incubated at 26 ± 2^0C and the spores were aseptically transferred to PDA plate for multiplication. Hyphal tip from the fungal colony grown on PDA was successively transferred to PDA medium following incubation at 26 ± 2^0C to get pure culture. The fungus was further sub cultured on Corn Meal Agar (CMA) media at 26 ± 2^0C to induce sporulation. The fungus grown on CMA medium was preserved in the refrigerator for further studies.

Isolation and preservation of effective strains of *Bacillus* spp.

Sixty six bacterial isolates were isolated and preserved from rhizosphere soil samples collected from different places of Jamalpur, Mymensingh, Tangail, Kishoreganj, Dhaka, Meherpur, Kushtia, Jessore, and Satkheera. For isolation of bacteria, 1 g of soil from each sample was added to 5 ml of nutrient broth and incubated at 35ºC for 24 hours (Amin *et al*. 2015). After incubation period, 100µL of the supernatant of each tube was inoculated by spreading a sterile glass rod on Carboxy Methyl Cellulose Agar (CMCA) plates (Kim *et al*. 2012).The CMCA inoculated plates were incubated at 28°C for 2 days. Bacilli were detected by clear halos around the colonies (Berlemont *et al*. 2009). The plates were examined and the expected colonies were selected for further streaking on Nutrient Agar (NA) medium plate to get pure cultures of *Bacillus* spp. Bacilli like colonies were roughly identified on the basis of their colony characteristics such as size, shape, color, margin and elevation. Pure cultures were obtained by repetitive streaking to get single colonies on NA plates. Plates were incubated for optimum growth for 48 hour at 28˚C (Mardanova *et al*. 2016). The *Bacillus* isolates preserved in glycerin solution in 1.5ml cryogenic vial at -80^0C for further study. In order to preserve, 750µl of NB culture supplemented with 750µl of 50% glycerol in a total volume of 1.5ml. For broth culture, the inoculated falcon tubes with NB were incubated in a rotary shaker incubator for overnight at 30^0C with 150 rpm.

***In-vitro* antagonism assay for selecting potential *Bacillus* spp.**

Potential *Bacillus* spp. were screened having antagonistic ability against *Magnaporthe oryzae* following dual culture technique on Corn Meal Agar (CMA).Disks of about 10 mm were cut from 7 days-old culture of *M. oryzae* and were placed on CMA plate and bacteria were streaked on the same plate a bit away from the mycelial block. After incubation at 24^0C for 5 days, the mycelial growth inhibition was evaluated following a scoring system. Bacteria over grown by hyphae scored as 0, hyphae at the edge (<0.5 cm) of the bacterial colony scored as 1 and a distinct inhibition zone (>0.5cm) around hyphae, bacterial colony scored as 2. Only those bacterial colonies that were able to induce a distinct inhibition zone (scored as 2) were selected for further characterization.

Growth suppression through dual culture assay

The effective bacterial isolates were tested for their ability to suppress the growth of *M. oryzae in vitro* dual culture method. Three replicates per treatment and three plates per replication were maintained in all cases and these plates were incubated at 24^0C for 7 days. The experiment was carried out following Completely Randomized Design (CRD).Data on mycelial growth (mm) were recorded at 2, 3 and 7 days after inoculation (Single line method).After incubation period, when the growth in control plate reached maximum, the radial mycelial growth of the pathogen was measured (Triangular method).Percent inhibition of mycelial growth was calculated using the formula (Vincent 1927)

$$Inhibition\,(\%) = \frac{C-T}{c} x100;$$

Where, C= Growth in control; T = Growth in treatment.

Molecular identification of *Bacillus* spp. by PCR amplification of 16S rDNA gene

Total DNA extraction of the isolates was done using PROMEGA kit following the manufacturer's instructions. The concentration of genomic DNA solution was determined at 260 nm by the following formula: DNA Concentration (ng/µl) =

$$\text{Absorbance reading} \times \frac{\text{Volume of deionized water (µµl}}{\text{Amount of DNA taken (µµl}} \times \text{Conversion Factor (0.05)x1000}$$

Working solutions of 50ng/µl DNA were prepared by adding the required amount of sterile distilled water (SDW) by following the formula:$V_1 \times S_1 = V_2 \times S_2$; The diluted DNA solution and the original stock were stored in a refrigerator at -20^{0}C for other experiments.

Polymerase Chain Reaction

The primer used in this experiment was 16SrDNA primer. The amplification of 16SrDNA gene was done by using universal bacterial 16SrDNA primer 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) and 27F (5′-AGAGTT TGATCTTGGCTCAG-3′) (Frank *et al*. 2008). The master mix of 25μL were composed of 1μL of bacterial culture as template DNA, 1μL of each primer (10μM), 12.5μL of 2xTaq PCR Master Mix (50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$.), and9.5μL of Nuclease-Free Water.The PCR consisted of primary denaturation for 5 min at 94°C; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 100 s; and an additional reaction for 10 min at 72°C. The amplified DNA obtained from PCR were utilized for electrophoresis on 1.25% agarose gel at 90V for 30 minutes. The gel was taken out carefully from the gel chamber and immersed on ethidium bromide solution for staining and finally placed on the UV trans-illuminator in the dark chamber for image documentation.

Nucleotide sequencing and phylogenetic analysis of 16S rDNA gene

The sequence of 16S rDNA gene was performed directly from PCR products according to the standard protocols for the ABI A3500 genetic analyzer (Applied Biosystems, Foster city, CA, USA) with BigDye$^{(R)}$v1.1 and 3.1 Cycle Sequencing Kits. The resulting sequences were compared with sequences found in Gen-Bank database using Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nih.gov/BlAST/). The quality of nucleic acid sequences was evaluated using Chromas (Version 2.6) software to avoid the use of low quality bases. The phylogenetic tree was constructed with sequences of bacteria in Genbank considering the highest homology. The phylogenetic tree was constructed by using (Phylogeny.fr) (Anisimova and Gascuel 2006).

Statistical Analyses

The collected data on radial mycelial growth were analyzed statistically by using MSTAT-C package program. The means for all the treatments were compared by DMRT (Duncan Multiple Range Test). The significance of the difference among the means was calculated by LSD (Least Significant Difference) test.

RESULTS

Morphological characters of *Magnaporthe oryzae*

During isolation from infected panicle of rice, we found two types of conidia *viz*. macroconidia and microconidia.

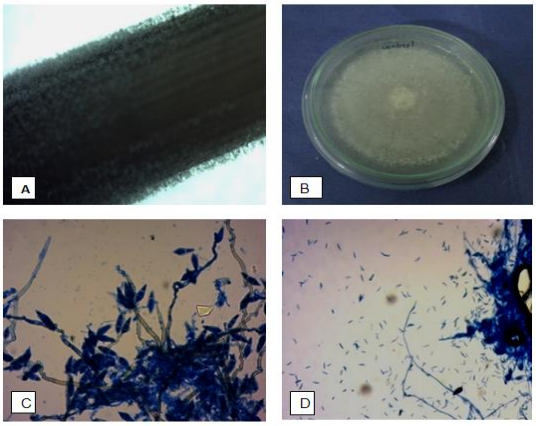

Plate 1. Morphological characters of *Magnaporthe oryzae*(A) *M.oryzae* on infected plant tissues on wet blotter paper (B) Pure culture of *M.oryzae* on Corn Meal Agar plate (C) Macroconidia- spindle shaped, hyaline, and three celled on wet blotter paper (D) Microconidia- crescent shaped, unicellular and hyaline on culture medium.

The macro conidia were pyriformed, three celled, hyaline and mostly profusely found on wet blotter paper. On the other hand, unicellular, hyaline, crescent microconidia were abundant on corn meal agar media culture plates. On culture medium the fungus exhibited smooth mycelial growth with dense velvet texture having greyish white color (Plate 1).

Initial screening of *Bacillus* spp. from rhizosphere soils

A total of 66 *Bacillus* spp. isolates were isolated on specific media(CMCA) from different locations of different agro-ecological zones of Bangladesh.In order to find out potential *Bacillus* spp. having growth suppressing ability against *M. oryzae* a triangular method of assay was followed (Score=0,1,2.).Among 66 initial*Bacillus* isolates, 36 isolates did not show any growth inhibition ability against *M. oryzae* (Score = 0), 19 isolates showed moderate inhibition (Score=1) and 11 isolates showed higher growth inhibition (Score=2) (Plate 2). The bacterial isolates showing higher inhibition (Score=2) were further studied for identification at molecular level and to find out the best isolates as a candidate for commercial formulation.

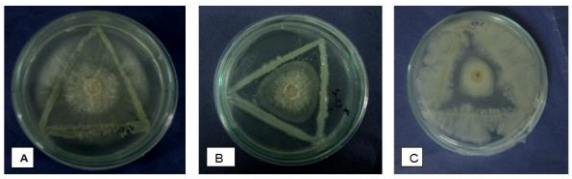

Plate 2. Scoring system during primary screening- (A) Bacteria overgrown by hyphae scored as 0, (B) Hyphae at the edge (<0.5 cm) of the bacterial colony scored as 1 & (C)A distinct inhibition zone (>0.5cm) around hyphae of bacterial colony scored as 2.

Growth suppressing ability of the selected isolates of *Bacillus* spp.(Single line method)

Initially nine *Bacillus* isolates were selected for further characterizing the growth suppression ability following dual culture assay (Single line method) against *M. oryzae*.Growth suppression ability was measured at 2,3 and 7 days after incubation. The radial mycelial growth of *M. oryzae* ranged from 10 to 78.18 mm in dual culture assay after 7 days of incubation. The average lowest growth (10.00 mm) of *M. oryzae* was recorded in dual culture assay plate with *Bacillus* isolates BSL-17, whereas the highest mycelial growth (78.18mm) was found in control plate (Table 1, Plate 3).Isolate BSL-10 also showed statistically significant growth suppression (82.80 % at 7 DAI) followed by BSL-21,BSL-15(ii) and BSL-31(Table 1).

Table 1. Mycelial growth of *M. oryzae* and %Inhibition by different bacterial isolates in dual culture assay (Single line method)

Sl No.	Name of isolates	Mycelial growth (mm)					
		2 DAI		3 DAI		7 DAI	
		Mycelial growth	% Inhibition	Mycelial growth	% Inhibition	Mycelial growth	% Inhibition
1	BSL-10	10.04 f	48.33	13.33 g	55.23	13.55 e	82.80
2	BSL-17	8.423 g	56.64	10.00 h	66.42	10.00 f	87.30
3	BSL-31	13.27 de	31.70	15.78 f	47.01	16.44 d	79.13
4	BSL-26(iii)	14.08 cd	27.53	17.11 d	42.54	17.41 d	77.90
5	BSL-21	12.37 e	36.33	15.89 ef	46.64	16.44 d	79.13
6	BSL-41	15.43 b	20.58	19.78 b	33.57	20.11 b	74.47
7	BSL-27(ii)	14.60 bc	24.85	16.56 de	44.39	17.00 d	78.42
8	BSL-15(ii)	13.00 e	33.09	16.56 de	44.39	16.89 d	78.56
9	BSL-8	14.27 cd	26.55	17.89 c	39.92	18.66 c	76.31
10	Control	19.43 a	----	29.78 a	-------	78.78 a	------
	LSD value	1.006		0.7114		1.010	
	CV (%)=	4.35		2.40		2.61	

DAI=Days After Incubation.

In a column means followed by a common letter are not different significantly.

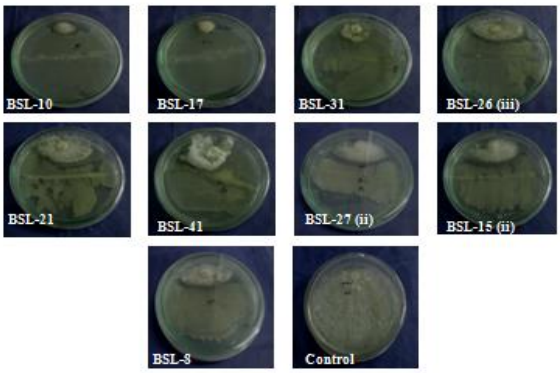

Plate 3. Growth suppression of *Magnaporthe oryzae* by selected *Bacillus* isolates in dual culture assay (Single line method)

Growth suppressing ability of some selected *Bacillus* isolates against *M. oryzae* (Triangular method)

To ensure the growth suppressing ability the previously tested *Bacillus* isolates were further examined in dual culture assay following triangular method. Based on the previous experiments, growth suppression data were recorded after 7 days after inoculation. At 7 DAI the lowest mycelial growth (12.30mm) was recorded in BSL-17 indicated 85.12% growth suppression compared to control (without *Bacillus*).Considerable growth suppression were recorded in BSL-10 (82.21%) and BSL-31(80.37%) (Table-2).

Table 2. Assessment of growth suppressing ability of some selected isolates of *Bacillus* spp. against *M. oryzae* (Triangular method)

SL	Name of the isolates	Mycelial growth (mm) (7 DAI)	%Inhibition over control
1	BSL-10	14.70 f	82.21
2	BSL-17	12.30 g	85.12
3	BSL-31	16.22 e	80.37
4	BSL-26(iii)	19.27 c	76.69
5	BSL-21	19.06 c	76.94
6	BSL-41	17.13 de	79.27
7	BSL-27(ii)	18.45 c	77.68
8	BSL-15(ii)	18.27 cd	77.90
9	BSL-8	20.80 b	74.84
10	Control	82.67 a	-----
	LSD value	1.187	
	CV (%)	2.90	

DAI= Days After Incubation
In a column means followed by a common letter(s) are not different significantly.

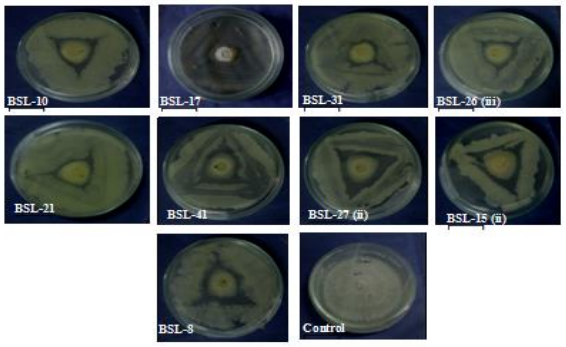

Plate 4. Growth suppression of *Magnaporthe oryzae* by selected bacterial isolates in dual culture assay (Triangular method) after 7 days of incubation

Identification of *Bacillus* Spp. isolates at molecular level

Amplification of 16s rDNA gene, the universal Primer,(27F and 1492R) were used.DNA samples of all the bacterial isolates were used during running Polymerase Chain Reaction (PCR) instead, one negative control (without DNA template).The amplified DNA obtained from PCR were utilized for electrophoresis in 1.25% agarose gel. A band of 1650bp was obtained on the lane 1-9, PCR without any template did not show any band (lane W) (Figure 1).

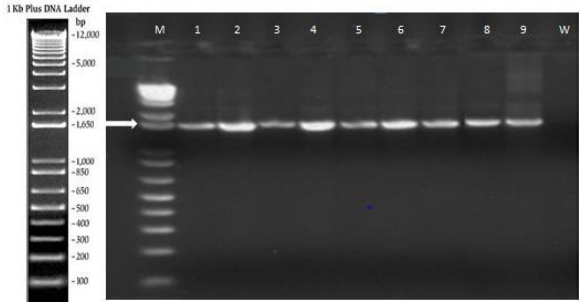

Figure 1. Agarose gel electrophoresis of selected bacterial DNA (16S rDNA) amplified with primer. Lane M-1kb plus DNA ladder; Lane1- Isolate BSL-10; Lane 2- Isolate BSL-17; Lane 3- Isolate BSL-31; Lane 4- Isolate BSL-26(iii); Lane 5-Isolate BSL-21; Lane 6- Isolate BSL-41; Lane 7- Isolate BSL-27(ii); Lane 8- Isolate BSL-15(ii); Lane 9- Isolate BSL-8; Lane W-Water. An amplicon size of 1650bp found after electrophoresis of PCR products.

Nucleotide sequencing and analysis

The nucleotide sequences with their translated amino acids done by amino acid translator software and the nucleic acid sequence quality found very well when evaluated using Chromas (Version 2.6) software. Best antagonistic isolate: BSL -17 showed 97% homology with *Bacillus subtilis* Bcx1 . Isolate: BSL -10 showed 93% homology with *I*

BSL -31 showed 96% homology with *Bacillus subtilis* Al-Khrj7, Isolate: BSL -26(iii) showed 95% homology with *Bacillus subtilis* MUST-2, Isolate: BSL -21 showed 95% homology with *Bacillus subtilis* BcX1, Isolate: BSL -41 showed 94% homology with *Bacillus subtilis* SH23 , Isolate: BSL -27(ii) showed 96% homology with *Bacillus subtilis* subsp. qingdao QND-8, Isolate: BSL -15(ii) showed 95% homology with *Bacillus subtilis* SH23, and Isolate:BSL-8 showed 81% homology with *Bacillus subtilis* BHR3P2B5-M (Table 3).

Table 3. Closest relatives of *Bacillus* spp. based on 16S rDNA sequences.

SL	Isolates(Location)	Close relatives	Accession	Location	Alignment	Homology
1	BSL-10 (Meherpur)	*Bacillus subtilis* TC-1	EU489517.1	China	843/908	93%
2	BSL-17(Mymensingh)	*Bacillus subtilis* Bcx1	JX504009.1	Changchun City, China	1292/1331	97%
3	BSL-31(Mymensingh)	*Bacillus subtilis* Al-Khrj7	KY123860.1	Riyadh , Saudi Arabia	1235/1289	96%
4	BSL-26(iii)(Jamalpur)	*Bacillus subtilis* MUST-2	KF727585.1	Pakistan	1391/1469	95%
5	BSL-21(Dhaka)	*Bacillus subtilis* BcX1	JX504009.1	Changchun City, China	1376/1453	95%
6	BSL-41 (Meherpur)	*Bacillus subtilis* SH23	KP735610.1	Viet Nam	1297/1374	94%
7	BSL-27(ii) (Kishoreganj)	*Bacillus subtilis* subsp. qingdao QND-8	KM234223.1	Shandong 266109, China	1335/1386	96%
8	BSL-15(ii) (Meherpur)	*Bacillus subtilis* SH23	KP735610.1	Viet Nam	1289/1357	95%
9	BSL-8 (Jamalpur)	*Bacillus subtilis* BHR3P2B5-M	KC849252.1	Odisha, India	1064/1314	81%

Phylogenetic analysis of 16SrDNA gene

The phylogenetic tree was constructed based on sequence of 16S rDNA gene of antagonistic bacterial isolates and compared with previously reported isolates of different places using Phylogeny.fr (Anisimova and Gascuel 2006).The length of Phylogenetic tree expressed the genetic distance of the isolates from the ancestors. The isolate-11 was much more distantly related to the ancestor than the other isolates. The value 0.5 meant the genetic distance of the isolates of each group. Phylogenetic tree analysis of the isolates based on the amino acid sequences of 16S rDNA gene revealed that all these *B. subtilis* isolates were distributed in two main groups. Isolate-11 belonged to Group I and there were 8 strains included in Group II. These strains were again grouped into two main clusters (Figure 2). Cluster I (Isolate-10, Isolate-4 and Isolate-3) and cluster II (Isolate-9, Isolate-6 and Isolate-2). The isolates of these two clusters were supported by bootstrap value of 75% (Figure 2). All the members of Cluster I were not very close to each other and generated one sub cluster consisting the isolates 10 and 4. That sub cluster was supported by bootstrap value of 93% (Figure 2). The bootstrap value is a value of statistical confidence in clustering of isolates during construction of Phylogenetic tree.

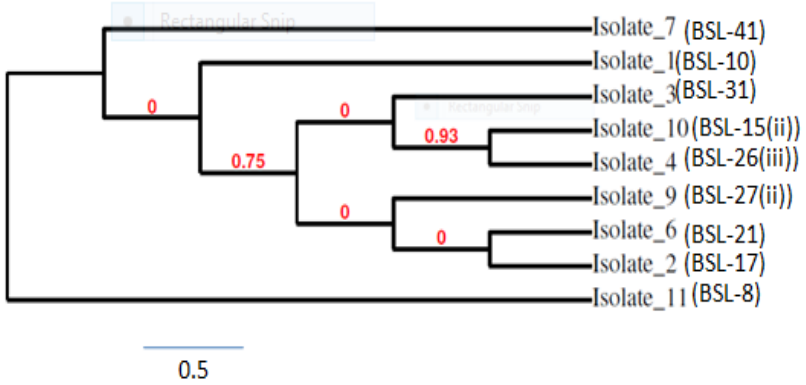

Figure 2. Phylogenetic tree constructed based on amino acid sequence of partial 16S rDNA gene of isolates of *Bacillus subtilis* (Phylogeny.fr) (Anisimova and Gascuel 2006).

DISCUSSION

The mode of action in biocontrol activities in *Bacillus* spp. is mainly through direct antagonism of Phytopathogen (Yu *et al.* 2002) and stimulation of plant disease system through the induction of systemic resistance (Choudhary and Johri 2009). Again *Bacillus* isolates can survive in adverse environmental conditions, resistant to high heat (80^0C), and high salinity (Perez-Garcia *et al*. 2011). *Magnaporthe oryzae* is an emerging phytopathogen in Bangladesh. Most recently the incidence and severity of rice blast in case of popular rice cultivars BRRI dhan28, BRRI dhan29, BRRI dhan34, BRRI dhan47 become very alarming. So, a durable and eco-friendly strategy is becoming more important.

The fungus *M. oryzae* produced two types of conidia. Macroconidia were spindle shaped, three celled and hyaline. And the microconidia were hyaline, crescent shaped and unicellular. This finding is strongly supported by Chuma *et al.* (2009).In dual culture assay among nine isolates the BSL-17 showed highest inhibitory effect against *M. oryzae.* The mycelial growth was 10mm in single line method and 12.30mm in triangular method which indicates strong inhibition in dual culture. In case of %inhibition, isolate-BSL-17 showed 85.12% to 87.30% mycelial inhibition against rice blast pathogen. This result is much better than the research findings of Subhalakshmi and Devi (2017). They found *P. fluorescens* B 24 giving maximum mycelial inhibition (77.5%) among the bacterial bio-control agents against rice blast pathogen.

There were clear inhibition zones (greater than 0.5cm), which were similar to those observed during the initial screening to identify effective isolates. The inhibition zone formed in dual culture assay was due to the secretion of antimicrobial metabolites (Kumar *et al*. 2012). Presence of inhibition zone during dual culture assay proved the presence of biologically active metabolites that diffused through the agar medium and protect the fungal growth (Yoshida *et al.* 2001).This research finding is also more or less similar to the findings of Yu *et al.* (2011). They examined the significant potentiality of *Bacillus subtilis* as biological control agent against 15 plant fungal pathogens including rice blast pathogen. The strain *B. subtilis* CAS15 showed strong antagonism in dual-culture with rates of inhibition ranging 19.26 to 94.07%.The present study revealed much better results compared with the result of Leelasuphakul *et al.* (2006). They identified *B. subtilis* NSRS 89-24 which resulted in approximately 60% inhibition of *P. grisea* in dual culture test. Similar work was conducted by Taguchi *et a*l. (2003) and the antagonist *B. subtilis* IK-1080 was cultured with the rice blast fungus on potato sucrose agar plates. They observed a significant suppression of hyphal growth of rice blast pathogen.

This study consists nine bacterial isolates with suppressive abilities against *M. oryzae*. For effective and antagonistic isolates, phenotypic and morphological characteristics and 16SrDNA sequence confirmed that all the isolates belonged to *Bacillus* genus and all the isolates were different strains of *Bacillus subtilis.* Sequencing of 16S rDNA is a powerful tool for identifying bacteria and determining phylogenetic, evolutionary relationship and classification of microorganisms (Weisburg *et al*. 1991).

Polymerase Chain Reaction (PCR) was performed for bacterial isolates with primer set 27F and 1492R to multiply 16S rDNA gene. The amplicon product gave an amplicon size of 1650bp. All the nine isolates showed positive in PCR amplification. The same research work was conducted by Liu *et al*. (2009) for molecular detection of *Bacillus subtilis.* The research work of Vijayalakshmi *et al*. (2012) aligned the sequence of 16S rDNA (1468bp) by using the BLAST program to identify the most similar sequence in the database of NCBI. They downloaded 16S rDNA sequences of different strains of *Bacillus subtilis* and its phylogenetically related species and genera from GenBank database and constructed a neighbour-joining phylogenetic tree using CLUSTALW algorithm with the help of MEGA software version 4.1. This research work is much coincided with the present research methodology.The result of nucleotide analysis in present study revealed that all the bacterial isolates were matched with different *Bacillus subtilis* strains of different places. Best antagonistic isolate: BSL -17 showed 97% homology with *Bacillus subtilis* Bcx1 from Changchun city in China. The phylogenetic tree showed that all the isolates were closely related to the *Bacillus subtilis* strains of different places of China, Saudi Arabia, India, Viet Nam and Pakistan.

Bio-control agents may or may not work in the field especially for panicle blast of rice. However, the present study is a first solid step towards developing a sound bio-control agent for this important disease of rice in Bangladesh. The findings of the present study ventilate the way of more investigation on identification and application of antimicrobial metabolites of these antagonistic bacteria. It is also important to develop suitable bacterial formulations with higher degree of stability and survival for the commercialization of bio-control agent.

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