Article

Probiotic *Bacillus* Species: Promising Biological Control Agents for Managing Worrisome Wheat Blast Disease

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Abstract: Plant diseases are among the major factors affecting plant productivity. Biological control of plant diseases is preferred over chemical control as it is environment-friendly, cost-effective, and sustainable. Among many microbes capable of providing biological control of plant diseases, probiotic *Bacillus* species are most promising as they can survive in adverse conditions, provide plants with a wide range of benefits including protection from phytopathogens. Wheat blast caused by *Magnaporthe oryzae Triticum* pathotype (MoT) has emerged as a potential threat to global wheat production. Due to unreliability of fungicides and limited cultivar resistance, we aimed to screen and identify potential antagonist bacteria collected from internal tissues of rice and wheat seeds to determine their *in vitro* and *in vivo* inhibitory effects against MoT. Dual culture and seedling assays were performed to evaluate the efficacy of probiotic bacteria. Out of 170 bacterial isolates, three bacteria (BTS-3, BTS-4, and BTLK6A) were screened as potential antagonists against MoT *in vitro*. Artificial inoculation at the seedling stage showed that the isolates BTS-4, BTS–3, and BTLK6A reduced 89, 88, and 85% of wheat blast disease severity, respectively, compared to mock-inoculated control. The bacterial isolates were identified as *Bacillus subtilis* (BTS-3) and *B. velezensis* (BTS-4 and BTLK6A) through genome phylogeny. The whole genome sequence of these three bacterial strains decoded a number of orthologs to intrinsic genes of antimicrobial peptides, antioxidant defense enzymes, cell wall degrading enzymes, compounds involved in induction of systemic resistance (ISR) in host plants and volatile compounds to make them promising biologicals to control MoT in wheat. Combined data of in vitro and in vivo along with genome analysis suggest that *Bacillus* spp. suppress the destructive wheat blast disease likely through antibiosis and ISR in the host plants. Further field evaluation and characterization of antimicrobial compounds are needed for better understanding of the mode of action and practical recommendation of these bacteria for wheat blast control in the farmers' fields.

Keywords: *Bacillus*; bacterial antagonist; genome sequence; antimicrobial peptide; biologicals

1. Introduction

Wheat (*Triticum aestivum* L.) is the world's most extensively cultivated cereal crop and provides 20-25% of the daily protein and calories to the consumers [1]. It is the main nutrient source for 40% of the world's population [2]. The consumption of wheat has been steadily increasing year over year. In 2020 – 2021 fiscal year, the worldwide wheat consumption was 759. 54 million metric tons, which was 12 million metric tons higher than

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the fiscal year 2019 - 2020 [3]. It is the second most important cereal crop in Bangladesh after rice. It plays an important role towards attaining food and nutritional security of the increasing population of this highly populated country. The wheat production was 1.08 million tonnes in 2021 in a fall from 1.30 million tonnes in 2014 according to Bangladesh Bureau of statistics (BBS) [4]. Both acreage and production of wheat had been increasing until a sudden massive outbreak of wheat blast occurred in 2016 [5]. In February 2016, wheat blast disease was spotted in Bangladesh for the first time in a country outside of South America that devastated more than 15,000 hectares of wheat with up to 100% yield losses [6], and very recently, it was also identified in Zambia [7] and potentially threatens wheat production in Europe [8]. After the epidemic of this pathogen in Bangladesh, it was rapidly identified by field pathogenomics and open data sharing against the aggressive clonal population of a South American lineage of *M. oryzae* (anamorph *Pyricularia oryzae*) *Triticum* (MoT) pathotype [6]. This finding created a worldwide concern that this deadly wheat killer may spread to neighboring countries of Bangladesh.

MoT, a filamentous and heterothallic ascomycete fungus, can infect more than 50 grass species. Immediately after infection, it blocks the vascular system, resulting white head symptom [9]. Application of chemical fungicides after head blast appearance is ineffective. Moreover, high reliance on chemical fungicides is not reliable and also negatively impacts the environment, soil, and human health. Fungicide treatments are expensive to resource poor farmers and poses risk of resistance development if used recurrently [10]. Development of resistant variety against wheat blast faces uphill battle due to the scarce of resistance genes identified so far [11]. In addition, plant resistance is likely to be less durable in field due to the evolution of new MoT races [12,13,14]. Therefore, development of effective biological control agent (BCA) together with other bio-rational options may be an efficient approach to control MoT.

Plant-beneficial microorganisms such as bacteria can be attractive natural alternatives of chemicals for biological control of destructive phytopathogens such as MoT [15]. Isolation of untapped beneficial bacteria from particular environments, such as internal plant tissues, is a new research approach [16,17]. Bacteria colonize inner plant tissues for all or part of their lifetime and promote the growth and fitness of host plants against biotic and abiotic stresses [18]. Like gut microflora in humans, bacteria inside plants exhibit complex interactions with their hosts and have been proven as a potential source of biocontrol agents [17]. Numerous studies have indicated that probiotic bacteria inhibit pathogenic growth directly by producing various primary [19] and secondary metabolites [17,20] or by inducing host systemic resistance (ISR) [21,22]. They also promote host growth through solubilization of nutrients [23,24], nitrogen fixation [25] and production of plant growth regulators [26]. Previous studies suggest that the probiotic bacteria can protect rice plants from blast fungus [27] and bacterial species under the genera of *Serratia*, *Pseudomonas* [28,29], *Streptomyces* [30,31], *Paraburkholderia* [32], and *Bacillus* [15,33,34]) have been reported as effective antagonists for biological control of rice blast fungus [6,35]. There are only a few reports describing that the pure compounds from some bacteria can suppress the growth of MoT *in vitro* [1,36,37,38,39]. However, no reports have been published on the biological control of wheat blast by seed endophytic bacteria.

To develop effective biologicals to control wheat blast, the specific objectives of the present study were to- (i) screen bacterial antagonistic to MoT from the seeds of local cultivars of wheat and rice grown in Bangladesh; (ii) evaluate the inhibitory effects of selected antagonists against MoT *in vitro* and *in vivo*; (iii) identify the potential bacterial antagonists through genome sequencing; and (iv) elucidate the underlying molecular mechanisms of antagonistic bacteria to control wheat blast.

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2. Results

2.1 Suppression of MoT mycelial growth by seed endophytic bacteria

2.1.1. Dual culture assay

Three potential bacterial isolates *viz*. BTS-3, BTS-4, and BTLK6A were identified as potential biocontrol agents against MoT in dual culture assay from screening of 170 bacterial isolates (**Figure 1**). All three bacterial isolates showed strong but differential inhibition of MoT hyphal growth (**Figure 1A**). The MoT inhibition rate by different bacteria significantly varied from untreated control ($F_{3, 16} = 217.25$, $p \leq 0.001$). The inhibition of MoT mycelial growth varied from 49.42% to 53.16% with the highest mycelial inhibition observed in BTLK6A (53.16%), followed by BTS-3 (50.71%) and BTS-4 (49.42%) (**Figure 1B**).

Microscopic analyses of MoT mycelial growth revealed that the untreated control MoT had regularly branched hyaline tubular hyphae with smooth and intact structures (**Figure 1A**). BTS-3 bacterial treatment induced excessive branching with pointed tips. However, BTS-4 induced swelling and disintegration of MoT hyphae along with excessive branching. Nodulation with hyper branching and tapering of MoT hyphal tips were found in MoT mycelia treated with BTLK6A bacterial isolate (**Figure 1A**).

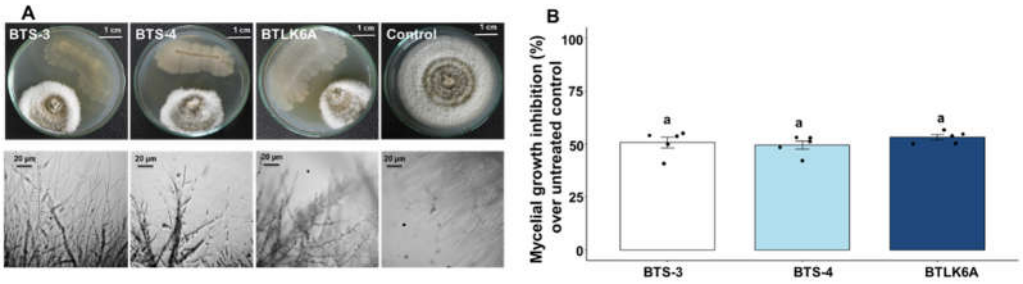

Figure 1. Inhibitory effects of probiotic bacteria on mycelial growth of MoT. (A) Micrograph representing the suppression of mycelial growth and induction of morphological alternations in MoT hyphae approaching the colonies of antagonistic bacteria in PDA; (B) MoT mycelial growth inhibition (%) by probiotic bacteria over untreated control. Data were recorded 14 days after inoculation and incubated at 25 ± 2 °C (n = 5, ANOVA with Tukey multiple comparison test, $p<0.05$; same letters on bars are not statistically significantly varied from each other). The black dots represent the data points for each bacterial isolate.

2.1.2. Cell-free culture filtrate of probiotic bacteria suppresses mycelial growth of MoT

The effect of cell-free autoclaved bacterial filtrates were tested to determine whether the antifungal activity of the bacterial isolates was due to the secretion of antifungal compounds in the liquid culture media. Bacterial cell-free culture filtrates significantly suppressed the radial growth of MoT (**Figure 2A**). The lowest radial growth (0.49 cm^2) was recorded in the culture plate containing cell-free culture filtrate of BTLK6A and it was almost similar (99.10%) to the radial growth inhibition by commercial fungicide Nativo (60 ppm) ($p = 0.73$). However, 97.56% (1.45 cm^2) inhibition of MoT radial mycelial growth was recorded for BTS-4 treatment, and 80.19% (11.79 cm^2) reduction was recorded for BTS-3 compared to untreated control (**Figure 2B**).

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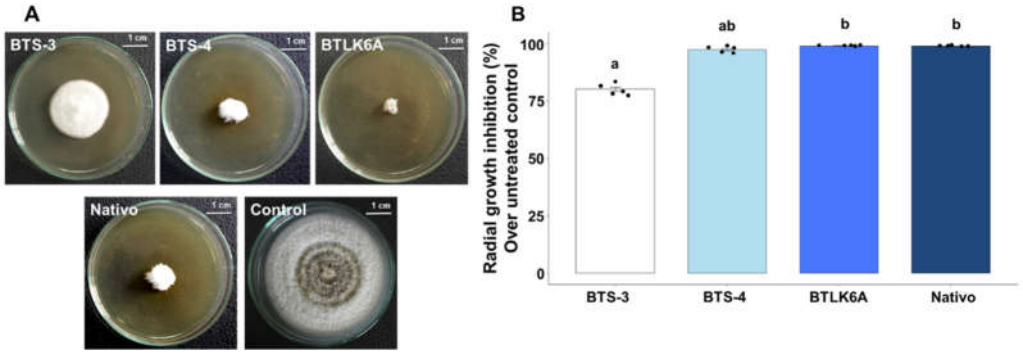

Figure 2. Inhibitory effects of bacterial cell-free autoclaved bacterial culture filtrates on MoT mycelial growth. (A) Inhibitory effects of probiotic bacterial cell-free culture filtrates on mycelial growth of MoT in PDA plates; (B) Inhibition of MoT mycelial growth (%) by bacterial cell-free culture filtrates over untreated control. Data were recorded 14 days after inoculation and incubated at 25 ± 2 °C (n = 5, Kuskal-Wallis test with Dunn multiple comparison test, chi-squared value = 22.3, $p \leq 0.001$; same letters on bars are not statistically significantly varied from each other). Black dots represent the data points for each bacterial isolate.

2.2. Assessment of biocontrol effects of bacteria on seedling assay

A pot experiment was performed to evaluate the efficacy of bacterial antagonists (BTS-3, BTS-4, and BTLK6A) against MoT *in vivo*. Eye-shaped blast symptoms were observed 3-4 days after MoT inoculation in both treatments pertaining to preventive and curative control measures. A significant variation in the biocontrol efficacy of each bacterial isolate was observed on seedlings under both control measures compared to untreated control. Furthermore, seedlings inoculated with antagonists as preventive measures showed lower disease severity compared to curative control, which is consistent with any biological control measure.

The reduction of seedling blast severity ranged from 84.66 to 89.31% in preventive control measures by bacterial antagonists. The highest reduction of seedling disease severity was recorded in BTS-4 (89.31%), followed by BTLK6A (88.25%) and BTS-3 (84.66%) (**Figure 3A–C**). However, the difference are statistically non-significant ($p = 0.472$) (**Figure 3D**). In curative control measures, the reduction of seedling disease severity ranged from 69.45 to 78.54%. Similar to preventive control, the highest reduction was documented for BTS-4 (78.54%), followed by BTLK6A (75.35%) and BTS-3 (69.45%) (**Figure 3D**).

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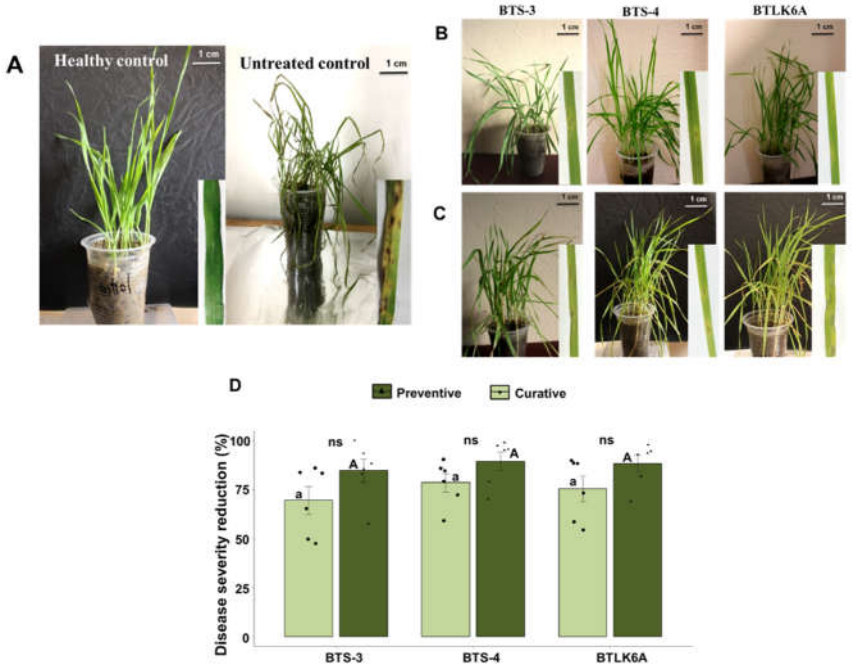

Figure 3. Suppression of leaf blast disease in wheat seedlings cv. BARI Gom-24 (Prodip) by endophytic antagonistic bacteria. (A) Seedlings of healthy control (no bacteria or MoT inoculation) and untreated control (plants only inoculated with MoT); (B) Effects of bacterial preventive control measure to control seedling leaf blast caused by MoT; (C) Effects of bacterial curative control measure to control leaf blast caused by MoT; (D) Reduction of leaf blast disease severity (%) in wheat seedlings by application of probiotic bacteria compared to untreated control. As a preventive control measure, wheat seedlings were sprayed with antagonistic bacteria 24 h prior to the inoculation of MoT conidia whereas wheat seedlings were sprayed with antagonistic bacteria 24 h after MoT conidia in curative control measure. Same letters on bars are not significantly different from each other; small letters on bars represent significant differences among curative bacterial control measures and capital letters on bars represent significant differences among preventive bacterial control measures. Non-significant differences (ns) observed in curative and preventive control measures of each bacterial isolate (ANOVA with Tukey multiple comparison test, n = 6, $p \leq 0.05$, each pot contained ten seedlings and treated as one replicate).

2.3. Identification of bacterial isolates

2.3.1. Morphological and physiological characterization of bacterial antagonists

All three bacterial isolate colonies were creamy white in color, positive for oxidase and catalase test, motile, and grew well in NBA medium supplemented with NaCl. The BTS-4 isolate showed the highest salt tolerance and grew well up to 10% NaCl concentration. However, only BTLK6A bacterial isolate could produce indole-3-acetic acid (**Table 2**).

Table 2. Morphological and physiological characterization of three potential bacterial antagonists against MoT.

Bacterial isolate	Source	Colony characteristic	Biochemical test										IAA
			KOH	Gram	Catalase	Oxidase	Motility	Salinity tolerance (%)					
								2	4	6	8	10	
BTS-3	Rice seeds cv. Rangabinni	White, sticky, round	-	+	+	+	+	+	+	+	+	-	-
BTS-4		White, sticky, round	-	+	+	+	+	+	+	+	+	+	-
BTLK6A	Wheat seeds cv. Kanchan	White, irregular	-	+	+	+	+	+	+	+	-	-	+

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2.3.2. Molecular identification of bacterial antagonists of MoT

Three selected bacterial isolates were identified as *Bacillus subtilis* BTS-3, *B. amyloliquefaciens* BTS-4, and *B. amyloliquefaciens* BTLK6A based on 16S rRNA gene sequencing earlier [40,41,42]. Changes on the genomic levels due to developing plant-associated life cycle, reinvestigation of our bacterial taxonomic status was performed following the phylogenomic analysis proposed by Dunlap *et al.* [43], and later they were identified as *Bacillus subtilis* BTS-3 (NCBI accession WOVJ00000000), *B. velezensis* BTS–4 (accession number WOVK00000000), and *B. velezensis* BTLK6A (accession number WOYD00000000).

The phylogenetic relationship of the selected bacterial isolates are shown in **Figure 4**. The phylogenetic tree revealed that BTS-4 and BTLK6A are closely related at the same node, and BTS-3 is distantly related to BTS-4 and BTLK6A.

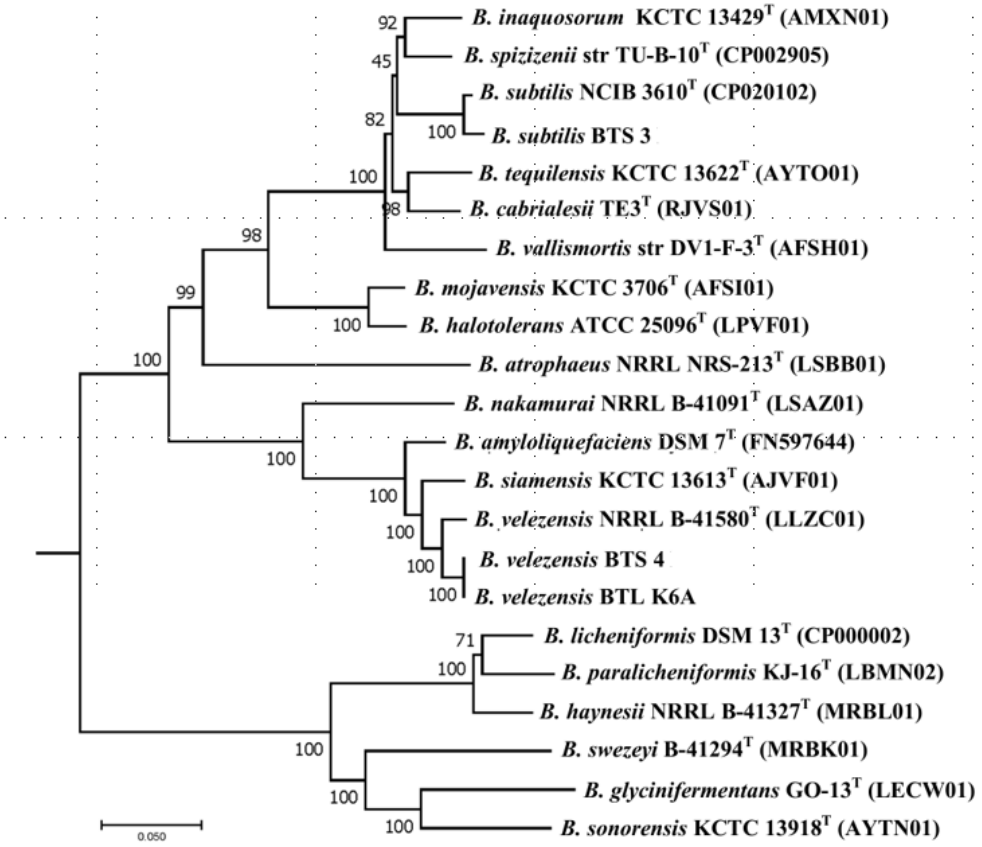

Figure 4. Phylogenetic neighbour-joining tree reconstructed from the core genomes of selected type strains of species from the *Bacillus subtilis* group. Bootstrap values 50%, based on 1,500 pseudoreplicates are indicated on brach points. *Bacillus indicus* was used as an out group, and only the relevant part of the tree is presented. The scale bar corresponds to 0.05 nucleotide substitutions per site.

2.4. General genomic features of antagonistic bacteria

The *de novo* assembly resulted in the estimated chromosome size of BTS-3 was 4,121,943 bp with 47 contigs. The overall G+C content, N_{50} and L_{50} values of the assembly were 43.5%, 1,063,829 and 2, respectively. The genome predicted 117 RNA genes, and 4,272 protein coding genes in putative functional categories. The largest contig assembled was 1,140,720 bp and the average coding sequence size was 849 bp (**Table 2**).

The estimated genome size of BTS-4 and BTLK6A was 3,907,662 bp and 3,908,699 bp with 27 and 32 contigs, respectively. The N_{50} values of the assembly were 2,032,688 and 1,024,542, whereas the L_{50} assembly value was 1 and 2, respectively. Both genomes predicted 113 RNA genes, protein coding genes (3,966 in BTS-4 and 3,968 in BTLK6A). The largest contig assembled were 2,032,688 and 1,083,238 bp, respectively and the average coding sequence size of BTS-4 and BTLK6A were 881 and 880, respectively (**Table 2**).

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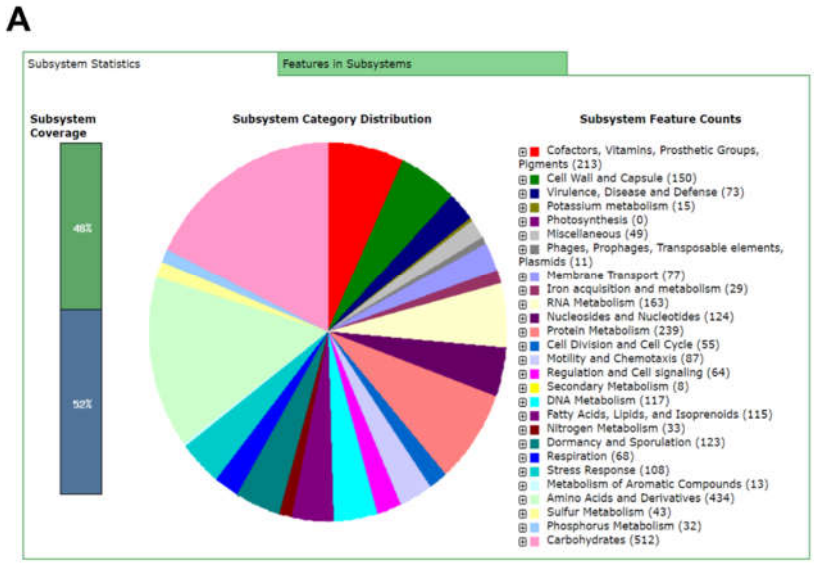
Table 2. General genomic features of bacterial antagonists of MoT.

Genomic Features	BTS-3	BTS-4	BTLK6A
Closest strain	*B. subtilis*	*B. velezensis*	*B. velezensis*
Genome size (bp)	4,121,943	3,907,662	3,908,699
Contigs	47	27	32
Largest contigs	1,140,720	2,032,688	1,083,238
G+C content (mol%)	43.5	46.5	46.5
N 50 (bp)	1,063,829	2,032,688	1,024,542
L 50	2	1	2
Protein-coding sequences	4,272	3,966	3,968
Percent of coding region	88.0	89.4	89.3
Average CDS size (bp)	849	881	880
Total number of RNAs	117	113	113
Number of Ribosomal RNAs	17	14	17
Number of tRNAs	85	84	84
Phage-associated genes	13	13	13
Number of Subsystems	477	463	463

2.4.1. Subsystem analysis of bacterial antagonists

Rapid annotation using subsystem technology (RAST) predicted 477 subsystems for BTS-3 bacterial isolate. Among those 73 subsystems are responsible for virulence, disease and defense; 87 for motility and chemotaxis; 8 for secondary metabolism; 29 for iron acquisition and metabolism and 64 for regulation and cell signaling (**Figure 5A**).

Both BTS-4 and BTLK6A predicted 463 subsystems. Both bacterial isolates contained 63 subsystems accountable for virulence, disease and defense; 85 for motility and chemotaxis; 12 for secondary metabolism; 30 for iron acquisition and metabolism and 65 for regulation and cell signaling (**Figure 5BC**).



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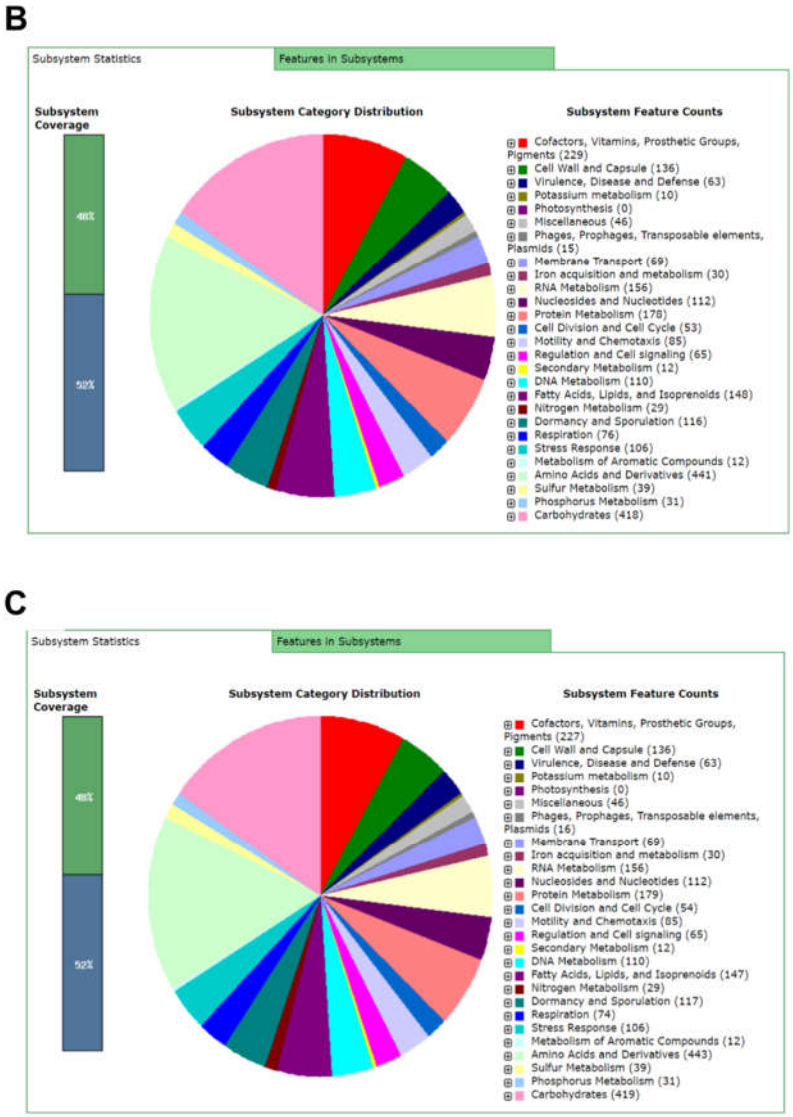

Figure 5. RAST server predicted subsystem categories for bacterial antagonists of MoT.

2.4.2. Bacterial genomic features for antagonism

The genome of BTS-3 encodes several orthologs of intrinsic genes of antimicrobial peptides, including bacillaene (*baeBCDEGHIJLMN*), bacilysin (*bacABCDEFG*), bacillibactin (*dhbABCE*), and fengycin (*fenABCDE*) (**Table 3, Figure 6**). The genome encodes several gene clusters related to antioxidant defense enzymes like- superoxide dismutase (*sodACF*), glutathione peroxidise (*bsaA*), catalase (*katAEX*) and thiol peroxidase (*Tpx*). Additionally, cell wall degrading gene clusters including esterase (*estAB*), endoglicanase (*eglS*), beta-glucanase (*bglS*), and pectatelyase (*pelABC*) were detected in the genome using BAGEL4. The presented genome also encodes for the gene cluster related to volatile compound acetoin (*alsSD*) (**Table 3**).

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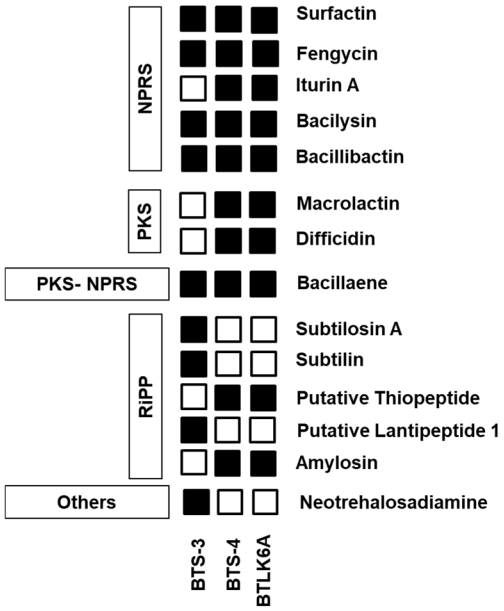

Figure 6. Antimicrobial peptides of potential bacterial antagonists of MoT.

The BTS-4 and BTLK6A contained 463 subsystems in their genomes and like BTS-3, BTS-4 and BTLK6A encodes the similar intrinsic genes of antimicrobial peptide with the addition of Iturin A (*ituABCD*), macrolactin (*mlABCDEFGHI*) and difficidin (*dfnABCDEFGHIJKLM*) genes (**Table 3, Figure 6**). The gene cluster related to antioxidant defense enzyme, cell wall degradation enzyme and volatile compounds in BTS-4 and BTLK6A were also similar to BTS-3 (**Table 3**).

Table 3. Gene clusters present in the synthesis of Antimicrobial peptides, antioxidant, cell wall degradation and volatile compound coding gene clusters present in BTS-3, BTS-4 and BTLK6A bacterial genome.

Compound	Enzyme	BTS-3	BTS-4	BTLK6A
Gene clusters related to antibiotic production				
Bacillaene	PKS/NRPS	*baeBCDEGHIJLMN*	*baeBCDEGHIJLMN*	*baeBCDEGHIJLMN*
Macrolactin	PKS	Not present	*mlnABCDEFGHI*	*mlnABCDEFGHI*
Difficidin	PKS	Not present	*dfnABCEFGHIJKLM*	*dfnABCEFGHIJKLM*
Bacilysin(siderophore)	NRPS	*bacABCDEFG*	*bacABCDEFG*	*bacABCDEFG*
Bacillibactin	NRPS	*dhbABCE*	*dhbABE*	*dhbABCE*
Fengycin	*NRPS*	*fenABCDE*	*fenABCDE*	*fenABCDE*
Iturin A	NRPS	Not present	*ituA*	*ituA*
Gene clusters related to antioxidant enzyme				
Superoxide dismutase		sodACF	sodACF	sodACF
Glutathione peroxidise		*bsaA*	*bsaA*	*bsaA*
Catalase		*katAEX*	*katAEX*	*katAEX*
Thiol peroxidase		*Tpx*	*Tpx*	*Tpx*
Gene cluster related to cell wall degradation				
Esterase		*estAB*	*estAB*	*estAB*
Endoglucanase		*eglS*	*eglS*	*eglS*
Beta-glucanase		*bglS*	*bglS*	*bglS*
Pectatelyase		*pelABC*	*pelAB*	*pelAB*
Gene cluster related to volatile compound				
Acetoin		*alsSD*	*alsSD*	*alsSD*

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3. Discussion

The invasive wheat blast fungus is a new serious threat to the food and nutritional security of Bangladesh, India, and other Asian wheat-growing regions [6]. Traditional approaches to control this troublesome disease is ineffective and breeding resistant variety requires several years although prospects of developing a variety with high durable resistance is bleak due to the unavailability of strong resistance source. This study attempted a novel strategy to develop a bio-rational management strategy using locally available beneficial plant bacteria from wheat and rice seeds.

In vitro dual culture assay displayed consistent inhibitory effects of seed endophytic bacteria against MoT. Three selected bacterial antagonists (BTS-3, BTS-4, and BTLK6A) showed considerable variability in the inhibitory activities against MoT. Interestingly, MoT growth inhibition by those antagonists caused morphological alternations of MoT hyphae, such as excessive branching, swelling, and cell disintegration in the approaching hyphae. The probable mechanisms behind the bacterial antagonism to MoT may comprise the production of secondary metabolites, biofilm production, secretion of lytic or cell wall degrading enzymes, and competition for space or nutritions [15,34]. Autoclaved cell-free culture filtrates from antagonistic bacteria similarly suppressed MoT growth on PDA. This culture filtrate assay suggested that the bacterial strains can produce thermostable extracellular antimicrobial compounds. Chakraborty *et al.* [1,36] reported that the linear lipopeptides from marine *B. subtilis* and oligomycins from *Streptomyces* spp. effectively controlled MoT pathogen. However, there are no detailed reports on rice or wheat-derived bacteria that successfully control wheat blast pathogen, MoT. A large body of literature is available on the biological control of rice blast by different probiotic bacterial isolates [33,34,44,45,46] and has been reported that the heat-stable cell-free culture filtrates from probiotics suppress the mycelial growth of rice blast [47,48]. A further study is warranted to characterize the thermostable antimicrobial compounds in the cell-free culture filtrates that effectively control the growth of MoT fungus.

The success of biocontrol agents largely depends on the survival and shelf-life of the microbial agents. Several lines of evidence suggest that the efficacy of BCAs were higher in the greenhouse and lower in field test due to the complex interactions with uncontrolled environmental factors including temperature [49,50,51]. In our current study, bacterial preventive and curative control measures effectively controlled wheat blast disease at seedling stage. In addition to disease suppression, bacterial antagonists also enhanced seed germination and growth of wheat seedlings (**Supplementary Figure 1, Table 1**). It was reported that the bio-inoculation of probiotic bacteria helps to promote plant growth [52,53]. Seed endophytic *Bacillus* spp. is not harmful to mammals [15,19] and application of *Bacillus* spp. induced genes related to auxin, gibberellin, jasmonic acid, salicylic acid production and upregulated MAPK signaling pathway of host to promote plant growth [54].

Our bacterial isolates were identified as *B. subtilis* BTS-3, *B. amyloliquefaciens* BTS-4, and *B. amyloliquefaciens* BTLK6A initially [40,41,42]. However, later on, those isolates were identified as *B. subtilis* BTS-3, *B. velezensis* BTS-4, and *B. velezensis* BTLK6A through whole genome sequencing. The BTS-4 and BTLK6A bacterial isolates were closely related (**Figure 4**). The genus *Bacillus* consists of 318 species [55]; *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, and *B. licheniformis* are phenetically and phylogenetically homogenous and combinedly known as *B. subtilis* species complex [56]. However during last few decades, many novel *Bacillus* species belonging to the *B. subtilis* species complex have been identified. Phylogenetic analysis through 16S rRNA gene fails to discriminate all the *B. subtilis* species complex. However, based on complete or whole genome sequencing, the *B. subtilis* species complex was discriminated accordingly [43].

Additionally, whole-genome mining revealed that all three selected *Bacillus* spp. possess gene clusters responsible for the biosynthesis of large array of antimicrobial compounds, cell lytic enzymes and compounds related to ISR. Macrolactin, bacillaene, bacilysin, bacillibactin, fengycin, and iturin A were major gene clusters predicted in

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bacterial genomes (**Table 3, Figure 6**). Biosynthesis and secretion of all these compounds by *Bacillus* spp. have shown direct mechanisms to suppress plant pathogens [57,58,59]. Several studies reported that the macrolide compounds macrolactin, bacillaene, and difficidin inhibit protein synthesis, impair cell division, and damage the cell membrane to restrict growth of phytopathogens [33,60]. Moreover, recent studies suggest that they also play a role in transcription modulation and enhance plant resistance against phytopathogens [15,61]. Siderophore such as bacilibactin, and bacilysin suppress fungal growth by competing for nutrition [62,63]. Interestingly, all three antagonist bacteria possess *fenABCDE* gene clusters responsible for the fengycin biosynthesis. This compound is also known as plipastatin, has been reported to damage cellular composition and organization by creating vacuoles in hyphae, thus inhibiting fungal growth [64]. Although three bacterial isolates used in this study possess a diverse range of antimicrobial peptide genes in their genome, it is necessary to investigate which compound is precisely effective against MoT.

Gene clusters related to oxidative stress have also been predicted in bacterial genomes. Gene clusters responsible for the biosynthesis of superoxide dismutase (*soda*, *sodC*, and *sodF*), hydrogen peroxide decomposing catalase (*katA, katE*, and *katX*), thiol peroxidase (*tpx*) and glutathione peroxidase (*bsaA*) have been predicted in our selected bacterial isoaltes. Under biotic and abiotic stress, various reactive oxygen species (ROS) are produced in plant cells and scavenged or detoxified by various antioxidant enzymes and metabolites. Hasanuzzaman *et al.* [65] described coordinated actions of osmoregulation, ion homeostasis, and antioxidant defense induced in host plants by the application of *B. subtilis*. The antioxidant enzyme-related gene clusters present in our biocontrol bacteria may be involved in ROS metabolism during blast infection.

Plant growth-promoting bacteria can trigger induced systemic resistance (ISR). All three biocontrol bacterial strains included in this study possessed gene acetolactate decarboxylase, *alsSD* gene cluster encoding acetoin biosynthesis. Several lines of evidence suggest that acetoin is a powerful elicitor to trigger induced systemic resistance in plants [66]. Yi *et al.* [67] demonstrated that volatile acetoin produced by *B. amyloliquefaciens* UCMB5113 significantly reduces infection of *Bipolaris sorokiniana* and promotes the growth of wheat seedlings compared with seedlings not exposed to bacterial volatiles before pathogen inoculation. The presence of gene clusters for the biosynthesis of acetoin in all of our *Bacilli* suggested that they induce systemic resistance in wheat plants and suppress wheat blast disease. Chowdhury *et al.* [68] also showed that acetoin produced by PGPR is effective in biocontrol of plant pathogens and its *in situ* expression takes place during root colonization.

Taken together, current genome analytical data coupled with *in vitro*, and *in vivo* bioassays unambiguously suggested that the BTS-3, BTS-4, and BTLK6A have diverse potentials to produce a wide range of antimicrobial compounds, cell wall degrading enzymes and induce systemic resistance to protect plants from MoT fungus. Isolates of *B. velezensis* BTLK6A, BTS-4, and *B. subtilis* BTS-3 have link with the production of characteristic antimicrobial compounds in concert with the induction of systemic resistance in wheat plants against blast fungus MoT. This is the first study that demonstrated wheat blast disease suppression by three seed endophytic native bacteria. Findings from the current study have opened a new window for further studies to discover novel clues for the biorational management of wheat blast. Therefore, it is necessary to identify and characterize anti-MoT substances produced by these antagonistic bacteria and elucidate their mechanisms of action. Additionally, large-scale field evaluation using this three potential *Bacillus* spp. (BTS-3, BTS-4, and BTLK6A) at reproductive stage of wheat are needed for recommending them as candidates for the formulation of biocontrol agents and to design integrated strategies [69] to control wheat blast disease.

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4. Materials and Methods

4.1. Bacterial strains and growth conditions

Total 170 bacterial isolates were obtained from the bacterial culture collection of Institute of Biotechnology and Genetic Engineering (BSMRAU), Bangladesh. The pure bacterial cultures were initially isolated from the seeds of traditional rice and wheat cultivars that were preserved in 20% glycerol at -20 °C and grown in nutrient broth agar, NBA (25 g in 1 litre, Sigma-Aldrich), for 24-48 h at 25 °C. Bacterial suspensions were prepared from the bacterial cultures in nutrient broth (NB) (3 colony inoculated in 250 ml NB in a 500 ml conical flask) incubated for 48 h in a rotary shaker (120 rpm) at 25 °C and then cultures were centrifuged (10,000 rpm for 10 min), and supernatant was discarded. The pellets were subsequently washed 3 times with sterilized distilled water (SDW) and bacterial concentration was adjusted at 1×10^9 CFU/ml.

4.2. Fungal isolate and culture conditions

Wheat blast fungal isolate BTJP-4(5) was also collected from the MoT culture collection of Institute of Biotechnology and Genetic Engineering (IBGE), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh. Dry paper discs containing blast fungal isolate were placed in Petri dishes containing potato dextrose agar (PDA) and incubated at 25 °C for 7 d. A 4 mm freshly grown 7 d old MoT mycelial plug was transferred to Petri dishes containing oatmeal agar (OMA) supplemented with Oracin K (250 mg Phenoxymethylpenicillin; 2 µg per 50 ml OMA, Sanofi Bangladesh Limited) for conidia production and incubated at 25 ± 2 °C for 7 d. After 7 d, the Petri dishes were irradiated 3 d under fluorescent lamps to induce MoT conidiation. To harvest MoT conidia, 10 ml SDW containing 0.02% (V/V) Tween 20 was added to Petri dishes and conidia harvested from the surface of MoT colony by a sterilized brush, followed by filtering the conidial suspension with sterilized cheesecloth, and the conidial density was adjusted 1×10^5 conidia/ml by using a hemocytometer (Fuchs- Rosenthal, 0.0625 mm^2) [70].

4.3. Antagonism assay

4.3.1. Dual culture assay

A single freshly grown bacterial colony was streaked in PDA (2 cm away from the edge of Petri dish), and a mycelial plug of MoT (6 mm) from the freshly grown 7 d old culture was placed at the opposite side of Petri dish (9 cm) perpendicular to the bacterial streaks and incubated at 25 ± 2 °C for 14 d [71]. The Petri dish containing only MoT mycelial plugs was used as a control, and five replications were maintained for each treatments. The percent mycelial growth inhibition was recorded 14 d after inoculation and calculated by the following formula: Mycelial growth inhibition (MGI) % = (C-T / C) × 100; Where, C = growth of MoT in control plate (cm), and T = growth of MoT mycelia in dual cultures (cm).

MoT hyphal morphologies in the vicinity of bacterial colonies were observed under a light microscope (Carl Zeies, Germany) and digital images were recorded by a digital camera (Canon EOS 700D, EF-S 18-55mm 3.5-5.6 IS STM).

4.3.2. Cell-free culture filtrate assay

A single bacterial colony from each bacterial isolate was incubated in a 100 ml Erlenmeyer flask containing 25 ml potato dextrose broth (PDB) and incubated for 24 h at 28 ± 2 °C. Then, 100 µl of each bacterial culture was inoculated in a 500 ml Erlenmeyer flask containing 250 ml of PDB, incubated at 28 ± 2 °C for 3 d at 120 rpm. Three days after incubation, the bacterial cells were removed by centrifugation (10,000 rpm for 10 min at 4 °C), and 10% bacterial autoclaved (121°C for 60 min) culture filtrates were used for preparing PDA plates. PDA plates containing Nativo fungicide (60 ppm) were treated as a positive control; plates containing only PDA without culture filtrates and fungicide were treated as absolute control. A 6 mm mycelial plug from 7 d old MoT culture was transfered at the

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center of each Petri dish and incubated at the same conditions described above (2.3.1). The radial MoT hyphal growth in each Petri dish was recorded at 14 d after incubation. For each treatment, 5 replications were used, and each experiment was repeated thrice.

4.4. Growing of seedlings

Wheat seeds cv. BARI Gom–24 (Prodip) was surface sterilized by following the protocol described by Paul *et al.* [38]. Fifteen seeds were sown in a plastic pot (12 cm × 7.5 cm) containing sterilized field soil amended with NPK fertilizer and grown for 15 days, maintaining 12/12 h light dark alternations and 65% humidity. Ten healthy seedlings were allowed to grow under natural conditions, and watering was done as needed.

4.5. In vivo assay for biocontrol activity of bacteria

Fifteen days after seedling emergence (DAE) two different experiments (preventive and curative) were conducted for seedling assay, and all pots were arranged in a completely randomized design in both experiments.

For preventive control assay, 100 ml of each bacterial suspension (ca. 1×10^9 CFU/ml) was sprayed on wheat seedlings at 15 DAE and left 24 h to dry. Twenty-four hours after bacterial inoculation, MoT conidial suspension (ca. 1×10^5 conidia/ml) was sprayed on the same wheat seedlings until the plant became wet and covered with polythene bags to maintain humidity (>90%) for 24 h at 25 °C in dark conditions to facilitate fungal infection.

For curative control measures, MoT spores (ca. 1×10^5 conidia/ml) were first spray inoculated in wheat seedlings and covered with polythene bags to maintain humidity (>90%) for 24 h at 25 °C in dark to facilitate fungal infection. Then, the seedlings were sprayed with bacterial suspension (ca. 1×10^9 CFU/ml). Both preventive and curative assays, the seedlings were then transferred to a growth chamber maintaining 25 °C temperature, 12 h light per day and >85% relative humidity. Wheat plants treated without MoT and bacteria served as healthy control, and plants treated with MoT alone without bacterial treatment served as untreated control. Sterilized water was sprayed on inoculated seedlings every day at 4.0 pm to maintain high humidity. Disease development was recorded at 7 d after inoculation, and each treatment was replicated six times [72,73].

4.6. Identification of antagonist bacteria

4.6.1. Phenotypic identification of bacterial isolates

Individual pure colonies of selected bacterial isolates grown in the NBA were carefully observed, and colony characteristics- colony type, size, colony color, and shape were recorded (Somasegaran and Hoben, 1994). A series of physiological and biochemical tests namely- KOH test, gram test, catalase, oxidase, motility test, indole acetic acid (IAA) production, growth in 2%, 4%, 6%, 8% and 10% NaCl were performed for phenotypic characterization of antagonistic bacteria following the methods described by [74,75].

4.6.1. Molecular identification of antagonistic bacteria

Potential antagonistic bacterial isolates were used for molecular identification. The genomic DNA from bacterial isolates was extracted by using a commercial GeneJET Genomic DNA extraction kit (Thermo Fisher Scientific, USA) The quality and extracted DNA concentrations were assessed by gel electrophoresis followed by comparing 1 Kb plus DNA ladder (Thermo Fisher Scientific, USA). The genomic DNA was used to construct a whole genome sequence library. The samples were fragmented with Covaris to around 550 to 600 bp, then the NEBNext Ultra DNA library preparation kit for Illumina was used (https://international.neb.com/products/e7370-nebnext-ultra-dna-library-prep-kit-for-illumina#Product%20Information). The constructed libraries were sequenced using Illumina HiSeq platform with 30.0x genome coverage. The raw data trimmed with Trimmomatic software and the quality assessed using in-house scripts combined with Samtools, Bedtools and bwa-mem softwares. The genome assembly was performed by using SPAdes (v. 3.10.0) method. The assembly matrix was calculated by using "QUAST"

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software. The taxonomic distribution of bacterial strains were determined by using Kraken software.

4.7. Construction of phylogenetic tree

Genome comparisons and alignments for phylogenetic trees were made using BIGSdb software [76]. The alignment used for the phylogenetic tree was based on the core genome of all strains found in the tree. MEGA X software was used to construct phylogenetic tree [77]. Neighbor-joining tree was reconstructed using the Tamura-Nei model [78] with a gamma correction (α=0.5) with complete deletion. This model was chosen on the basis of the likelihood test implemented in MEGA X. Measures of bootstrap support for internal branches were obtained from 1,500 pseudoreplicates.

4.8. Identification of metabolic genes responsible for antagonism

NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used for annotation of predicting protein coding genes, rRNAs, tRNAs. Best-placed reference protein set annotation method was used for annotating genome data by using GeneMarkS-2+ software (version 6.1). Rapid annotation using Subsystems Technology (RAST FIGfams v.70) was used to predict the open reading frames of a genome. Prodigal program was used to predict potential genes in a genome, and Blastp program was used to find out the similarities of predicted proteins against Uniprot protein database. Metabolic cluster and finding metabolic model was performed by antiMASH software .

4.9. Statistical analysis

Statistical data analysis was performed using R software (version 4.1.2). Linear regression models (LMs) were used for analyzing the data sets. The model fit for LMs was evaluated by using "DHARMa" package. The Shapiro-Wilk normality tests (shapiro.test function) was performed to determine whether the response variables met test assumptions. Analysis of variance (ANOVA) followed by Tukey multiple comparison ($p<0.05$) was performed using "emmeans" package for normally distributed data and Kruskal-Wallis test using "kruskal.test" function followed by Dunn multiple comparison analysis using "FSA" and "rcompanion" packages ($p<0.05$) for non-normally distributed data sets. The plots were visualized by using "ggplot2" function.

5. Conclusions

Three plant bacteria *viz. B. velezensis* (BTS-4 and BTKL6A) and *B. subtilis* (BTS-3) were identified and characterized as potential candidates for biological control of wheat blast through laboratory and greenhouse assays. The whole-genome sequence data revealed that these three bacteria contain different antimicrobial, cell wall degrading, induced systemic resistant and antioxidant enzyme-related genes clusters which potentially play an important role in antagonism and suppression of MoT growth in host plants. Further studies are needed to precisely identify the specific genes involved in the production of antimicrobial substances and also characterize the structural features of the chemical arsenals produced by those bacteria. A large-scale field evaluation of the efficacy of biocontrol bacteria at reproductive stage of wheat is needed before recommending them for practical use as biocontrol agents against wheat blast fungus in the practical field.

Supplementary Materials: Table S1.

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