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Research Article

Characterization of broad-spectrum biocontrol efficacy of *Bacillus velezensis* against *Fusarium oxysporum* in *Triticum aestivum* L.

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Abstract

Fungi are the most important phytopathogens that cause yield losses. The mycotoxins released by fungi cause spoilage of stored food consumed by humans and feed supplied to animals. Fungi-antagonistic microbes are gaining attention as potential biocontrol agents (BCAs). This study was designed to isolate bacterial isolates from different crops and evaluate their *in vitro* antifungal assay against three phytopathogens, plant growth promoting (PGP) characteristics, molecular identification, and *in vivo* efficiency against the most devastating phytopathogenic fungus *Fusarium oxysporum* Schltdl. In the *in vitro* experiment, the 3 isolates BA, GL-1, and 5a out of 360 isolates showed more than 60% inhibitory activity against the selected fungi in this study. On the basis of 16S rRNA sequencing and phylogenetic analysis, BA isolate was identified as *Bacillus velezensis*. All three isolates produced indole acetic acid (IAA), hydrogen cyanide (HCN), and cellulase enzymes, while the BA and GL-1 isolates also produced siderophores and the BA isolate also produced ammonia. BA was selected on basis of not only Biocontrol efficacy but also maximum PGPR activity compared to GL-1 and 5a. *In vivo assay,* the isolate BA showed a significant decrease in disease severity caused by *Fusarium oxysporum* by 64.97% after 100 days of inoculation on wheat (FD-08) seedlings in a greenhouse assay and enhanced the shoot root height, fresh and dry mass. The wide-ranging antagonistic action of *Bacillus velezensis* isolated from the phyllosphere of wheat crops showed promising fungicidal and plant growth-promoting capabilities, suggesting it can be used as a biofungicide.

Keywords: antagonistic; antifungal; green house; plant growth promotion; phyllosphere; *Triticum aestivum* L.

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Introduction

Phytopathogenic fungi cause diseases and catastrophic threats to plant health. Rice, wheat, maize, barley and other important crops are vulnerable to different fungal species, ultimately causing low yields, which lead to increased prices and food insecurity for humans and livestock (Mitchell *et al.*, 2016). The widespread soil-borne phytopathogen *Fusarium oxysporum*, a difficult fungus to control, belongs to Ascomycota, whose members infect approximately 150 different plant species and 36 hosts in Pakistan (Syed *et al.*, 2015; Rana *et al.*, 2017). Fusarium attacks growing seeds, juvenile plants and ruptured tissues, enters through roots and hinders the vascular system of plants, mainly causing blight and rot, and some species also produce mycotoxins (Toppo and Naik, 2015; Rana *et al.*, 2017). Fusarium wilt in cotton, chickpea and banana (Panama disease) is associated with significant economic losses, with an estimated reduction in yield of approximately 50% (Amanda, 2015; Dita *et al.*, 2018). Fusarium causes symptoms such as *Fusarium* seedling blight (FSB), root rot, and Fusarium head blight (FHB) on wheat crops (Pastuszak *et al.*, 2021).

The soil-resident fungus *Rhizoctonia solani* J.G. Kuhn is a basidiomycete found in almost all geographic regions and causes disease in different important crops (Ajayi-Oyetunde and Bradley, 2018; Khan *et al.*, 2019). On the basis of its different disease-causing mechanisms and genetically divergent groups, *R. solani* is called an anastomosis group (AG), and approximately 14 different AGs have been reported (Tewoldemedhin *et al.*, 2006). Widespread sheath blight caused by *R. solani* in rice causes serious yield loss from 25 to 50%, and all cultivars of rice are susceptible, although the degree of susceptibility differs.

Alternaria alternata (Fr.) Keissler, a phytopathogenic fungus belonging to division Ascomycota, is the causative agent of a wide range of pre- and postharvest plant diseases, including crop, fruit, cereal and vegetable diseases, across the globe, with approximately >300 species that can spread via spores and decaying plants (Leiminger *et al.*, 2015; Zhu *et al.*, 2017). This fungus has seven variants called pathotypes, which produce the host-specific toxins AF toxin and AK toxin, responsible for causing black spot on strawberry and Japanese pear, respectively (Tsuge *et al.*, 2013). Sunflower and soyabean leaf blight caused by Alternaria accounts for major yield losses, including approximately 80% seed loss and 33% oil yield loss in Pakistan and across the world (Kgatle *et al.*, 2018).

To overcome fungi, the apparent method of choice is the use of synthetic fungicides. The main adverse effects of chemical fungicides are elevated acute and chronic mammalian nontarget and environmental toxicology and later resistance and weakening of efficacy facilitating pest reappearance (Yoon *et al.*, 2013). Carboxylic acid- and glucopyranosyl-containing fungicides attack DNA topoisomerase II, and amino acid formation of fungi can also affect beneficial microbes in other eukaryotic cells (Yang *et al.*, 2011).

It is not a novel concept to use living natural systems in agriculture for plant protection and growth promotion; this approach can be traced back a long way in human history. In contrast, the need for such biocontrol agents is greater than before since concerns about the use of these compounds are persistent. The direct impact includes the uptake of insoluble nutrients, inorganic phosphate solubilization, mineralization of organic compound phytohormone production and nitrogen fixation. The indirect impacts are that biocontrol agents produce antimicrobial peptides (AMPs), metabolite production, enzyme production, antibiotics and siderophores, which can restrict the plant loss caused by plant pathogens (Velivelli *et al.*, 2015; Lucero *et al.*, 2021). The process of selecting microorganisms and commercialization of biocontrol products for phytopathogens is difficult because there are several standards to be analysed that are critical for the achievement of consequent steps. Mechanisms of antagonistic efficacy, growth that maintains an adequate population, and even studies of legal property rights and market penetration are vital (Fernandes *et al.*, 2021).

The agriculture sector is Pakistan's second largest, accounting for 24% of total GDP. The productivity of major crops such as wheat, maize, rice, sugarcane, and cotton are critical to the country's economic development (Shafi *et al.*, 2022). The purpose of this study was to evaluate broad-specturm antagonist activity of indigenous isolates against fungi and in vivo antagonistic activity against the phytopathogenic fungus *Fusarium oxysporum* on wheat variety FD-08 under greenhouse conditions.

Materials and Methods

Phytopathogenic fungi collection and maintenance

The fungi *Alternaria alternata, Fusarium oxysporum*, and *Rhizoctonia solani* used in this study were kindly provided by the Agriculture Research Service, United States Department of Agriculture (ARS, USDA), and maintained on PDA (potato dextrose agar) medium at 25± 2 °C for further use.

Isolation of bacteria with antagonistic activity against phytopathogenic fungi

A total of 360 samples from the phyllosphere and rhizosphere of different crops, including maize, potato, apple, pomegranate, barley, cotton, grapes, and wheat, were collected from different fields of Quetta, Baluchistan, Pakistan and processed at Applied Biotechnology Laboratory, Balochistan University of Information Technology, Engineering and Management Sciences (BUITEMS). The phyllosphere samples were processed as described by Ramzan *et al.* (2016), and rhizosphere samples were processed as described by Tsegaye *et al.* (2019). By using the spread plate method, 1 ml of each 10^{-6} CFU/ml diluted sample was spread on Luria Bertani agar (LBA) media, and plates were incubated at 28±2 °C for 72 hours. The bacterial colonies were sub-cultured and assessed for antagonistic activity against phytopathogens. They were further screened for different PGP traits. The bacterial strains showing antagonistic activity against phytopathogenic fungi were kept at -80 °C in glycerol stock.

In vitro antagonistic assays

Qualitative assays

The *in vitro* capability of bacterial isolates to stop the growth of phytopathogens (*A. alternata*, *R. solani* and *F. oxysporum*) was qualitatively assessed by the agar plug method, in which 5 mm agar discs of 7-day-old fungal pathogens were separately inoculated in the middle of petri-plates containing medium (PDA), fresh bacterial isolates were inoculated on three sides of the PDA plates (at least 2.5 cm distance) that were previously inoculated with phytopathogenic fungi, and plates were kept at 27 ± 1 °C for 5-7 days (Haidar *et al.*, 2016).

Quantitative assays

Quantitative assessment was performed by the dual culture method of Haidar *et al.* (2016). A 6 mm disc of 7-day-old fungi was placed in the middle of a plate containing medium (PDA), a fresh bacterial strain at 2.5 cm from the fungal disc in a straight line was co-inoculated on the same plate, and the plates were incubated for 5 days at 27± 1 °C. Agar plates inoculated only with fungi were kept as controls. The experiment was replicated in triplicate. The inhibition percentage was calculated by the formula

$$\text{Inhibition \%} = \{100 \times (\frac{C-T}{C})\}$$

where C= circular growth of fungus (phytopathogen) on control petri plates, and T = the circular growth of fungus (phytopathogen) in the presence of antagonistic bacterial isolates (Dennis and Webster, 1971; Azeem *et al.*, 2022).

Molecular identification of strains BA, 5a and GL1

Genomic DNA from freshly cultured cells was extracted using a Thermo-ScientificTM mini kit (K0721) as stated by the manufacturer's protocol. Using the 1.3 kb universal primers U1492R (5′-GGTTACCTTACGACTT -3′) and 27F (5′- AGAGTTTGATCMTGGCTCAG -3′), bacterial isolates underwent 16S rRNA gene amplification (Awais *et al.*, 2017). Amplifications were performed in a thermal cycler (Gradient Thermocycler, Biobase, China) in a total volume of 20 µl containing 2.5 mM $MgCl_2$, 1 mM of each dNTP, 0.1 mM of each primer, 2.5 µl of *Taq polymerase* (Thermo Scientific) and 50 ng of genomic DNA. The thermal cycler protocol was as follows: initial denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 57 °C for 1 minute and extension at 72 °C for 1.5 minutes, with a final extension at 72 °C for 10 minutes. The amplified product was resolved in agarose gel

and visualized on a Gel Doc system (Biobase China). A Thermo Scientific Kit (K0691) was used to purify the amplicon according to the manufacturer's protocol. The purified amplicon was commercially sequenced at Macrogen (Korea). The inferred sequences were deposited in the GenBank database, and accession numbers were obtained. The identification of phylogenetic neighbours and calculation of highly analogous sequences were carried out by using the National Center for Biotechnology Information (NCBI); similarly, the phylogenetic tree was created in MEGA-X by using the first 50 BLAST hits (Ramzan *et al.*, 2016).

Characteristics of plant growth promotion traits

The plant growth-promoting characteristics of the selected isolates *B. velezensis, B. amyloliquefaciens*, and *B. subtilis* were evaluated for phosphate solubilization, production of IAA, cellulose enzymes, siderophores, ammonia and HCN. All the experiments were conducted in triplicate.

Phosphate solubilization

National Botanical Research Institute phosphate growth medium (NBRIP) was prepared for the assessment of phosphate solubilization (Nautiyal, 1999). The pH of the NBRIP agar was adjusted to 7.0, and the three potential antagonistic isolates were inoculated separately in the centre of plates containing NBRIP medium. NBRIP plates without bacterial inoculation served as controls. The plates were kept at 37 °C for 72 hours, and the formation of clear halo zones was observed.

Production of indole 3-acetic acid (IAA)

A single pure colony of each potential isolate was aseptically inoculated in sterile LB broth (100 ml) with the addition of 0.1 g of L-tryptophan (Sigma Aldrich) to assess IAA production (Sivasankari, 2016). The flasks were incubated at 37 °C for 48 hours with shaking at 100 rpm. Three replications were maintained, and uninoculated LB broth was kept as a control. The centrifugation of cultures was performed at 13,000 rpm (DWB-D2012plus) for twelve minutes, and the supernatants were collected. The supernatants (1 mL) were reacted with 2 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% v/v per chloric acid) and incubated at 28 ± 2 °C for 30 minutes in the dark. The change in colour from colourless to pink-red indicated IAA production.

Screening for carboxymethyl cellulase (CMCase)

CMC agar medium was used to grow the bacterial colonies for CMCase screening, and the medium contained K_2HPO_4 1 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, NaCl 0.5 g/l, $FeSO_4.7H_2O$ 0.01 g/l, $MnSO_4H_2O$ 0.01 g/l, NH_4NO_3 0.3 g/l, carboxymethyl cellulose 10 g/l and agar 12 g/l. The bacterial isolates were streaked in CMC agar plates, and the plates were incubated for 96 hours at 30 °C. The plates were then flooded with gram iodine for 20 min and destained with 1 M NaCl for 20 min on a shaker at 50 rpm. A zone around the bacterial isolate after destaining was observed for cellulose production (Passari *et al.*, 2016).

Screening for siderophore production

The bacterial isolates were inoculated in broth (peptone 10.0 g/l; K_2HPO_4 1.5 g/l; glycerol 5 ml; $MgSO_4$, 15.0 g/l) for 96 hours at 28 ± 2 °C. The broth cultures were centrifuged at 20,000 rpm for 7 minutes, and the cell-free portions were collected and mixed with an equal volume of chrome azurol S solution (Schwyn and Neilands, 1987). This solution was incubated in the dark for 35 min. A change in colour from blue to orange showed a positive result for siderophore production (Jacob *et al.*, 2016). Three replications were maintained, and uninoculated broth served as a control.

Ammonia production

The antagonistic potential bacterial isolates were inoculated in peptone broth and incubated with shaking at 120 rpm for five days at 28 ± 2 °C. After that, the culture broth was centrifuged at 10,000 rpm for

five minutes. Approximately 1 mL of Nessler's reagent (0.09 molL^{-1} K2[HgI4] with 2.5 molL^{-1} KOH) was mixed with 9 mL of supernatant. The change in colour from brown to yellow showed the production of ammonia, and broth without Nesseler's reagent served as a control (Jacob *et al.*, 2016).

Production of hydrogen cyanide (HCN)

HCN production by the isolates was evaluated by the Lorck method (Lorck, 1948). The isolates (BA, GL-1, 5a) were grown in bennet agar (yeast extract 1 g/l, beef extract 1 g/l, casein enzyme hydrolysate 2 g/l, dextrose 10 g/l and agar 18 g/l) supplemented with glycine (4.4 g/l). Filter paper (Whatman grade 1 filter paper) was flooded with a solution consisting of picric acid (0.5%) added to 2% sodium carbonate for one minute. The plates were sealed with parafilm and incubated at 28 ± 2 °C for five days. The alteration from yellow to orange against filter paper indicated an isolate was positive for the production of HCN. Three replications were performed for each bacterial isolate.

In vivo PGP and antagonistic assay of Bacillus velezensis on wheat

The effectiveness and antagonistic effect of the isolate *Bacillus velezensis*, based on *in vitro* efficiency, was also evaluated for *in vivo* antagonistic and PGP assays in a greenhouse against phytopathogenic fungi (*Fusarium oxysporum*) on wheat (FD-08), and the indices were examined by using the process of Vasebi *et al.* (2013) and Passari *et al.* (2016) with slight modifications. Plastic pots (10 x 8 x 8 cm) were filled with pasteurized loam, perlite and peat moss (1:1:1). Wheat seeds (FD-2008) were obtained from the plant stress physiology laboratory, BUITEMS Quetta, surface sterilized for two minutes in 70% ethanol followed by 2% sodium hypochloride for five minutes and rinsed with dH_2O five times. Inocula containing the phytopathogen *Fusarium oxysporum* was prepared by growing in PDB at 25 ± 2 °C for 8 days. The culture was filtered with 4 layers of sterile chelate cloth, and the final concentration (10^6 CFU ml^{-1}) was adjusted using haemocytometer in sterile normal saline. *Bacillus velezensis* was grown in a 500 ml flask containing 200 ml of sterile LB broth at 30 ± 2 °C for 48 hours in a shaking incubator (Biobase, China) at 100 rpm, and a final concentration of 10^8 CFU ml^{-1} was maintained. The bacterial counting was done using serial dilution and subsequently dilutions were spread on LB agar medium. Seeds were soaked for 4 hours and kept on a shaker at 100 rpm in the suspension described in Table 1.

After 4 hours, the aseptic seeds were sown in seedling pots with sterile loam, perlite and peat moss. The treated seeds were sowed in the greenhouse. The trial was accomplished with 4 treatments with 3 replications each. A total of 5 mL of *Bacillus velezensis* at a concentration of 10^8 CFU mL^{-1} in the form of a soil drench was applied to T2 and T3, and *Fusarium oxysporum* at a concentration of 10^6 CFU ml^{-1} was applied to T2 to promote disease development. The bacterial and fungal inoculation treatment was repeated after seven days. Three grams of urea was given per pot two times during the experiment for each treatment. Harvesting was performed after 100 days, and root and shoot length, fresh weight and dry mass as well as root and shoot number were recorded and compared among the treatments (Passari *et al.*, 2016).

Each plant was rated using the following scales: 0 = no symptoms, 1 = less than 25% of plants with symptoms, 2 = 26 to 50% of plants with symptoms, 3 = 51 to 75% of plants with symptoms, and 4 = 76 to 100% of plants with symptoms.

Table 1. Treatment design for evaluation of *Bacillus velezensis* against *Fusarium oxysporum* in greenhouse conditions

S. No	Suspension	Concentration
T1	(1% CMC) + Normal Saline +seeds	----
T2	(1% CMC) + Normal Saline containing BA and pathogen + seeds	10^8 CFUmL^{-1}, and 10^6 CFUmL^{-1}
T3	(1% CMC) +Normal Saline containing isolate BA + seeds	10^8 CFUmL^{-1}
T4	(1% CMC) + Normal Saline containing pathogen + seeds	10^6 CFUml^{-1}

T1 = Mock control; T2 = *Bacillus velezensis* and *Fusarium oxysporum*; T3 = *Bacillus velezensis* only; T4 *Fusarium oxysporum* only

The percent disease index was calculated according to Passari *et al.* (2016):

$$DI\% = [R\ (rating \times number\ of\ plants\ rated) / (Total\ number\ of\ plants \times highest\ rating)] \times 100$$

Similarly, the vigour index of wheat plants in each treatment was measured by using the following formula (Ji *et al.*, 2014):

$$Vigour\ index = (Mean\ shoot\ length + Mean\ root\ length)\ Germination\ \%)$$

One-way ANOVA was used for statistical analysis at $P < 0.05$. Three replications in the greenhouse were accomplished in all trials with eleven plants per treatment.

Results

Selection of bacteria with antagonistic activity

Twenty bacterial isolates showed positive antifungal activity against all three fungi (*Alternaria alternata, Rhizoctonia solani* and *Fusarium oxysporum*) of the 360 isolated bacteria. Table 2 shows the colony characteristics of the twenty antagonistic bacterial isolates.

Table 2. Colony characteristics of antagonistic bacterial isolates and parts of crops used for isolation

Sl. No.	Isolates	Colony morphology	Source of samples	Gram staining	Spore staining
1	NB	White Circular dry	Maize leaves	+	NSB
2	NWP	White Irregular dry	Maize leaves	+	SB
3	BA	White Creamy irregular	Wheat leaves	+	SB
4	NB2	White Circular dry	Apple leaves	-	NSB
5	GL5	White creamy dry	Cotton root	+	NSB
6	E	White Dry irregular	Cotton leaves	+	SB
7	A	White Circular smooth	Cotton roots	-	NSB
8	GL-1	White Irregular mucoid	Grapes leaves	+	SB
9	SOP	White Dry irregular	Grapes leaves	+	SB
10	SRS	White creamy dry	Apple leaves	+	NSB
11	NWR	White creamy dry	Pomegranate leaves	+	NSB
12	NW6	White Rhiozoid dry	Pomegranate leaves	+	SB
13	6a	White Circular mucoid	Pomegranate leaves	-	NSB
14	4a	White creamy	Wheat root	+	NSB
15	5a	White Dry irregular	Barley root	+	SB
16	CAP	White Rhiozoid dry	Barely leaves	+	NSB
17	CAR	White creamy dry	Wheat root	+	NSB
18	MMR	White creamy dry	Maize root	+	NSB
19	MML	White Circular smooth	Maize leaves	-	NSB
20	CPR	White, Dry circular	Maize root	+	SB

(+) gram-positive bacteria, (-) gram-negative bacteria, NSB= non-spore-bearing bacteria, SB= spore-bearing bacteria

Qualitative assay

The 20 selected isolates were qualitatively assessed against the three phytopathogenic pathogens (*A. alternata*, *R. solani*, and *F. oxysporum*) by using the agar plug method (Table 3) as described earlier. Out of 20 isolates with antifungal activity, these three isolates (*B. velezensis, B. amyloliquefaciens*, *B. subtilis*) showed very strong antagonistic efficiency against the tested fungi.

Table 3. Qualitative assay of the isolates against phytopathogenic fungi

S. No.	Isolates	*Alternaria alternata*	*Fusarium oxysporum*	*Rhizoctonia solani*
1	NB	++	+	++
2	NWP	+	++	+
3	BA	++++	++++	++++
4	NB2	++	+	++
5	GL5	++	++	+
6	E	+++	+++	+
7	A	++	+	+
8	GL-1	++++	+++	++++
9	SOP	+	+	+
10	SRS	+++	+	+
11	NWR	++	++	++
12	NW6	+	+++	+
13	6a	+++	+	++
14	4a	++	+	+++
15	5a	++++	++++	++++
16	CAP	+	++	+
17	CAR	+	+	+
18	MMR	+	+	++
19	MML	+++	+	++
20	CPR	++	++	+

(++++) indicates very strong activity; (+++) indicates strong activity; (++) indicates moderate activity and (+) indicates weak activity

Quantitative assay

The isolates that showed positive antifungal activity were quantified using the dual culture method. All 20 isolates showed antagonistic activity ranging from 24-79%, as shown in Figure 1. Among 20 isolates, BA, GLI and 5a showed very strong antagonistic activity against the three tested phytopathogenic pathogens such as *A. alternata*, *F. oxysporum*, and *R. solani* as shown in Figures 1 and 2. All three isolates were gram-positive; BA and 5a were spore-bearing, while Gl-1 was nonspore-forming. BA, Gl-1 and 5a were characterized for molecular identification and for *in vitro* plant growth-promoting traits.

Molecular identification of the biocontrol bacterial isolates

BLAST examination using the 16S rRNA gene sequence showed that the BA, GL-1 and 5a isolates were aligned (98-99.11%) to *Bacillus* isolates. Using the neighbour-joining method, the evolutionary history was inferred (Saitou and Nei, 1987), and the optimal tree of the current study is shown in Figure 5. The replicate tree percentage is shown next to the branches where the associated taxa clustered together in the bootstrap test (1000 replicates)(Shimodaira and Hasegawa, 2001). Using the maximum composite likelihood method, the evolutionary distances were computed in units of the number of base substitutions per site. This analysis involved 50 nucleotide sequences. The ambiguous sites were detached for each sequence pair through the pairwise deletion option. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). Using the neighbour-joining dendrogram, phylogenetic analysis indicated that BA is closely related to *B. velezensis*. Gl-1 and 5a are related to *B. amyloliquefaciens* and *B. subtilis*, respectively.

Characteristics of plant growth promoting traits

The bacterial isolates BA, GL-1 and 5a, which showed very strong antifungal activity against the tested phytopathogenic fungi, were selected for characterization of *in vitro* PGP (plant growth-promoting) traits, as summarized in Table 4.

Indole-3 acetic acid (IAA) production and phosphate solubilization

The hormone indole-3 acetic acid (IAA), which is a vital hormone for plant growth promotion, tested positive in all three selected isolates by using Salkowski's reagent supplemented with tryptophan. Using the tryptophan-dependent pathway method, qualitative estimation of the results showed IAA production in the presence of tryptophan. None of the isolates solubilized phosphate.

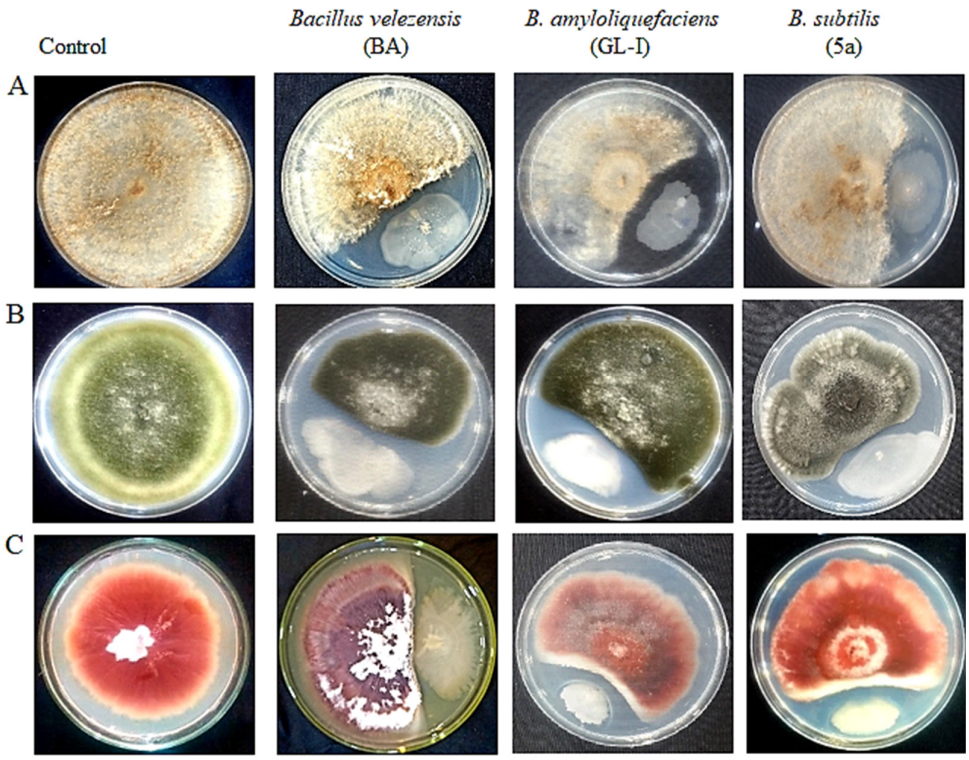

Figure 1. Qualitative antifungal assay of the three selected isolates

The left side shows the control. A) Antagonistic activity of BA, GL-1 and 5a against *Rhizoctonia solani* B) Antagonistic activity of BA, GL-1 and 5a against *Alternaria alternata* C) Antagonistic activity of BA, GL-1 and 5a against *Fusarium oxysporum*.

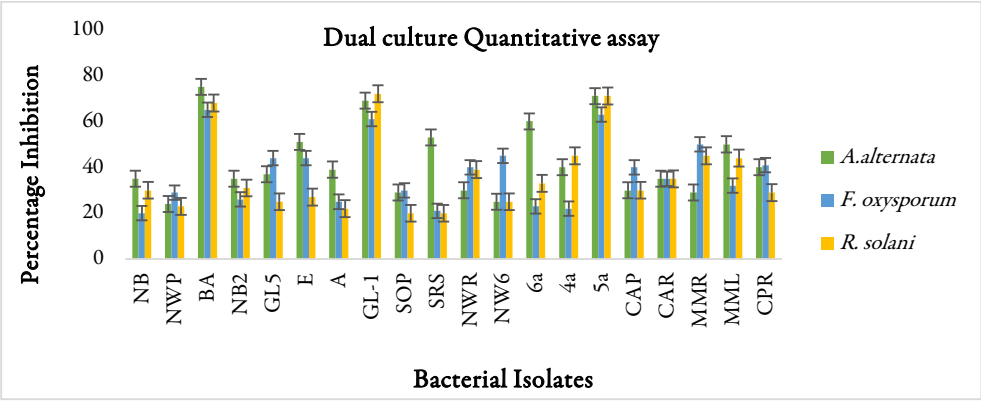

Figure 2. Graphical representation of the suppression of phytopathogenic fungi as a percentage against biocontrol isolates

The X-axis represents the bacterial isolates, while the Y-axis shows the per cent inhibition of fungi. Bacterial isolates were tested against *Alternaria alternate*, *Fusarium oxysporum*, and *Rhizoctonia solani*, and the bars represent the standard error of the mean.

Table 4. Summary of plant growth-promoting traits produced by the selected bacterial isolates

Isolates	Characteristics					
	Siderophore	HCN	Cellulase	Ammonia	PS	IAA
BA	+	+	+	+	-	+
GL-1	+	+	+	-	-	+
5a	-	+	+	-	-	+

HCN: Hydrogen cyanide; PS: Phosphate solubilization; IAA: Indole acetic acid. (+/-) indicates positive and negative results, respectively.

Cellulase (CMcase) and ammonia production

All three isolates showed cellulase production on CMC agar by forming a clear zone around the bacterial colonies. Among the isolates, GL-1 showed the largest zone (30 mm), followed by BA (19 mm) and 5a (16 mm). For ammonia screening, all the selected isolates (BA, GL-1 and 5a) were tested qualitatively for ammonia production. The isolate BA showed a positive result for ammonia production. The medium colour changed to brownish, indicating a positive result for ammonia production.

Hydrogen cyanide (HCN) and siderophore production

All antagonistic isolates (BA, GL-1 and 5a) tested positive for hydrogen cyanide production. The result was based on colour intensity developed by soaking filter papers. On CAS agar medium, siderophore production was detected in all tested isolates (BA, GL-1 and 5a) by forming an orange zone around the colonies. Isolate BA produced the largest zone (13 mm), followed by 5a (11 mm) and GL1 (9 mm).

In vivo antagonistic assay of a selected isolate

The bacterial isolate BA (*Bacillus velezensis*) was selected for the greenhouse experiment because it showed very strong antifungal activity *in vitro* against the tested fungi but also has better plant growth-promoting activity than other isolates and is a spore former. The phytopathogen *Fusarium oxysporum* was chosen for this experiment, as this fungus is relatively more lethal to wheat than the other fungi mentioned earlier. The *in vivo* biocontrol capability of *Bacillus velezensis* against *Fusarium oxysporum* showed that the isolate *Bacillus velezensis* reduced the disease rate significantly (64.97%) compared to pathogen treatment only, as shown in Figure 3. The symptoms such as necrosis, yellowing and wilting was reduced significantly by Bacillus velezensis compared to control.

A significant rise in root length, shoot length, fresh weight and dry mass after 100 days of inoculation (DOI) was observed, as shown in Figure 4. The bacterial strain BA displayed significant PGP (plant growth-promoting) activity in terms of shoots and root length ($p < 0.05$) when compared with the pathogen-inoculated control (T4) and comparable to the uninoculated negative control (T1). In addition, the BA isolates were competent to produce IAA, ammonia, HCN, and siderophores and showed higher antifungal activity against all tested phytopathogens, including *Fusarium oxysporum in vitro*.

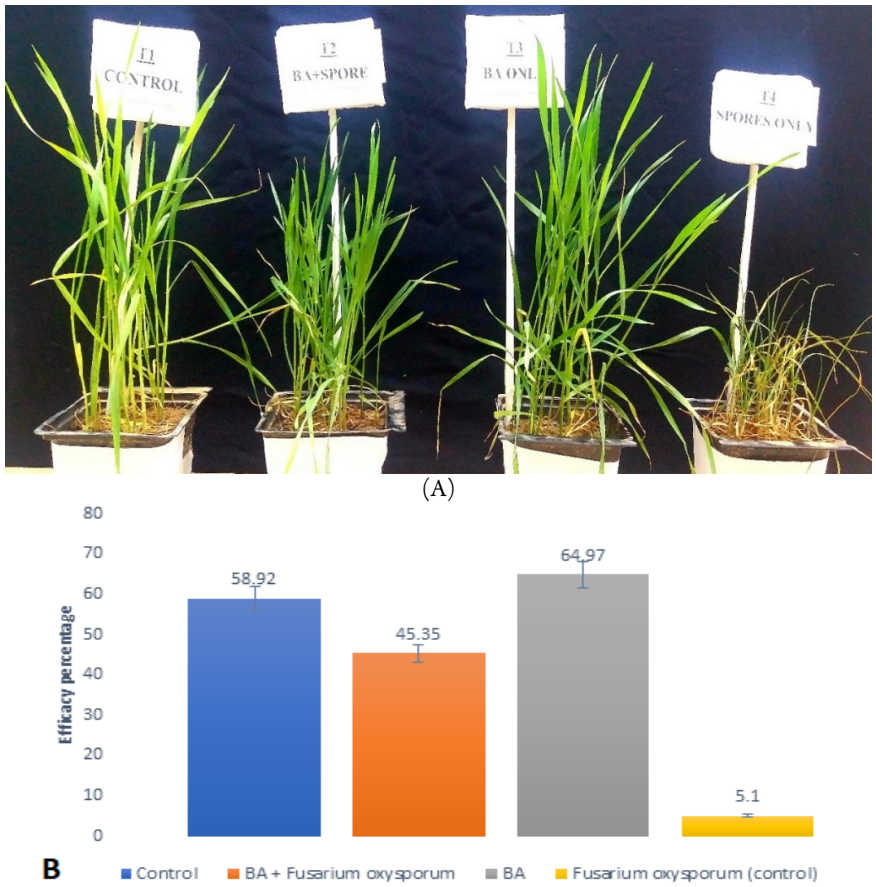

Figure 3. A) Greenhouse assay of isolate *Bacillus velezensis* on wheat (FD-08) against *Fusarium oxysporum*. T1: Mock control; T2: *Bacillus velezensis* and *Fusarium oxysporum*. T3: *Bacillus velezensis* (BA); T4: *Fusarium oxysporum* (spores) only; B) efficacy of *Bacillus velezensis* to control *Fusarium oxysporum*

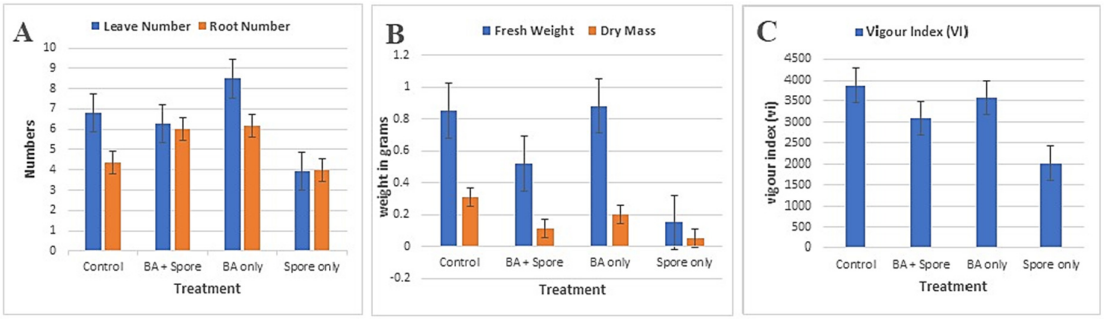

Figure 4. Plant growth-promoting trait of *Bacillus velezensis* on wheat FD-08 in greenhouse conditions after 100 DOI

A) Shoot and root length of FD-08; B) Fresh and dry mass weight of FD-08; C) vigour index of FD-08. The bar represents the standard error mean.

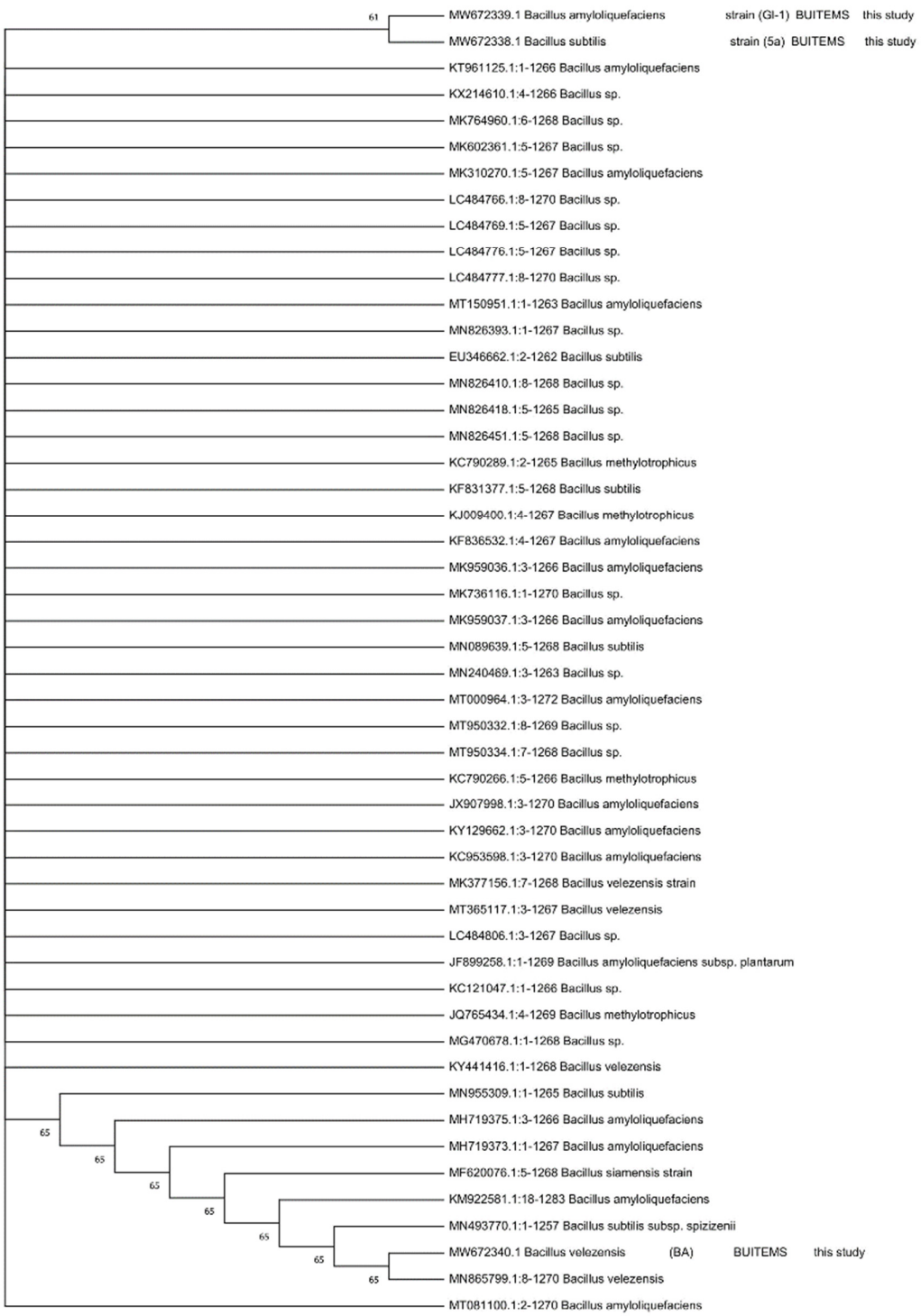

Figure 5. A dendrogram of *Bacillus* species was constructed using the 16S rRNA gene sequences acquired from the BLAST analysis and the sequences of the isolates used in this study

The number at each branch is the percentage of time the group of strains in that branch occurred, based on 1000 cycles in bootstrap analysis with MEGA-X.

Discussion

Pathogens in soil, such as bacteria, fungi, viruses and nematodes, cause major economic losses to crop. To overcome this constraint, synthetic chemical compounds are routinely applied to crops, which not only causes environmental contamination but also health problems for humans consuming chemically affected crops. The practice of applying insecticides or pesticides is undeniably a current exercise to control phytopathogens. However, it is no longer a defensible approach for the control of pathogens, as they are emerging with resistance to fungicides and pesticides. This has encouraged researchers around the world to have increasing interest in biocontrol agents. Presently, there is no target site for the isolation of potential bacteria that could provide resistance against plant diseases, although the suitable location for finding such a bacterial strain is analogous to that for the target pathogen. It has been reported that synergic factors are required for biocontrol efficiency, such as the characteristics of biocontrol agents, the epidemiology of the target pathogen and the environmental conditions.

The goal of this research was to characterize the efficacy of bacterial isolates from the rhizosphere and phyllosphere that would provide resistance against phytopathogenic fungi *in vitro* and *in vivo* and promote the growth of crops.

In *in vitro* assay, out of 360 bacterial isolates, 20 were screened and found to possess antagonistic efficiency at some level against all three fungi (*Alternaria alternata, Fusarium oxysporum*, and *Rhizoctonia solani*) used in this study. Qualitative antagonistic activity through agar plug method was the first line of screening, and all isolates that had no antagonistic activity against phytopathogens were eliminated. Furthermore, three bacterial isolates (*B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis*) were subjected to quantification assays against the fungi used in this study. The three bacterial isolates were selected based on very strong antifungal inhibition against all three fungi. The inhibition zone was determined in an *in vitro* study, which may suggest the release of fungicidal metabolites by potential bacterial isolates.

The 16S rRNA gene sequences of isolates BA, GL-1 and 5a displayed 98-99% homology with reference sequences in GenBank of NCBI. The BA isolate is gram-positive and spore-bearing; despite the fact that all three bacterial isolates have strong antagonistic efficiencies against the tested phytopathogens, BA (*Bacillus velezensis*) was chosen for *in vivo* study based on PGP activity of Ammonia production, which was not observed in GL-1 and 5a (*Bacillus amyloliquefaciens* and *Bacillus subtilus*). The same type of bacterium with PGP and biocontrol activity was isolated from the rhizosphere of rice soil by Madhaiyan *et al.* (2010). Studies have shown that *Bacillus* strains can be used as biocontrol agents for plant disease (Gajbhiye *et al.*, 2009), and our bacterial isolate BA showed 98.66% similarity to *Bacillus velezensis*.

For plant growth and development, phytohormone production is a significant element of plant growth and development of biocontrol isolates. IAA production by isolates allows plants to develop a well-grown root system, which raises the plants' nutrient uptake. In our study, the three isolates (BA, GL-1 and 5a) tested positive to produce IAA using l-tryptophan, indicating their genetic makeup is responsible for the variation production of IAA by multiple pathways. Previous studies found the same results and reported that bacterial isolates belonging to the same genus can produce different amounts of IAA (Gilbert *et al.,* 2018). In this study, none of the three tested isolates showed phosphate-solubilizing activity on NBRIP agar medium by establishing clear halo zones around the bacterial colony. Similar results were obtained by Jacob *et al.* (2016), who showed promising findings from a GH (greenhouse) study, but the isolate was unable to demonstrate phosphate solubilizing activity.

Ammonia production by bacteria can act as an activating factor by suppressing plant pathogens and is responsible for indirect plant growth through the accumulation and supply of nitrogen to the plant. Consequently, root and shoot elongation are stimulated, which eventually increases plant biomass. The isolate BA produces ammonia. These findings are consistent with those of Joseph *et al.* (2012), who reported ammonia production in 95.0% *Bacillus* spp.

The production of hydrogen cyanide (HCN) is another vital attribute of PGP, which plays a substantial role in combatting disease. The host plant is usually not affected by HCN generated by bacteria. It can operate as a biological control agent, whereas cyanide acts as a metabolic inhibitor to discourage competition predation or act as a safeguard from phytopathogenic fungi. All isolates in this research study (BA, GL-1, 5a) tested positive for HCN production. These findings are similar to Ghodsalavi *et al.* (2013), who found that approximately 90% of *Bacillus* spp. Are capable of producing HCN.

The role of siderophores in plant-bacteria association is to mobilize various metal ions, such as Fe, Cu and Zn (Tabli *et al.*, 2018). Siderophores may excite the *b*iosynthesis of other antimicrobial compounds directly by enriching the accessibility of minerals to bacteria that may stop the development of phytopathogenic fungi as well as other pathogenic microorganisms. The present study showed that the isolates BA and GL-1 were positive for siderophore production. The isolate BA (*Bacillus velezensis*) produced siderophores that are in agreement with Pereira Castro-Silva (2010), who reported that *Bacillus subtilis* (BS-8), isolated from rhizospheric soil, exhibited a high capacity for siderophore production.

Cellulase play a significant role in disease suppression but also helps in plant growth elevation and organic matter decomposition. In the current work, all three isolates (BA, GL-1 and 5a) exhibited positive cellulase production by forming a clear halo zone around the bacterial colonies on CMC agar, verifying the studies of Vipul *et al.* (2012) and Da*s et al.* (2010), which showed that all *Bacillus* spp. In the experiment were positive for cellulase production.

In case of *in vivo* experiment, the isolate *B. velezensis*reduced *Fusarium oxysporum* disease symptoms by 64.97% in a greenhouse environment based on biocontrol observations and a significant vigour index compared to the control. According to Wang *et al.* (2020b), *Bacillus velezensis* BM21 showed antifungal activity against *F. graminearum*. Similarly, Jiang *et al.* (2019) used *Bacillus velezensis* F21 to inhibit *Fusarium oxysporum* in watermelon under greenhouse conditions. Analogous outcomes described by Yigit Dikilitas (2007) and Passari *et al.* (2016) reported that *Pseudomonas fluorescence* and *Bacillus* Sp. repressed *Fusarium oxysporum* growth in tomato plants under greenhouse conditions. According to Wang *et al.* (2020a), *Bacillus velezensis* FKM10 not only inhibited *F. verticillioides* but also enhanced the plant growth of *Malus hupehensis* R. Biocontrol bacteria displayed activity on a widespread range of agricultural crops, facilitating growth development and increasing crop yield, disease control, and stress tolerance. Research was performed to assess the competence of isolates and to screen the plant growth stimulating factors of *Bacillus* spp. using an *in vitro* antagonistic approach. The isolate (BA) involved in the elevation of growth in a greenhouse setting showed a significant antagonistic effect and an increase in root growth, shoot length and dry mass of wheat (FD-08) seedlings compared to the control after 100 days of seedling growth. The current outcome was significant ($p < 0.05$) when related to the control. According to Jasrotia *et al.* (2021), *Bacillus* spp. not only inhibited *Magnaporthe oryzae*, causing blast of rice but also significantly enhanced the shoot and root length of basmati rice. Several studies have also shown that endophytic bacteria can increase the root and shoot length in various plants, and such a significant effect on the growth and development of plants may compensate the host plant with respect to overall growth (Eid *et al.*, 20021).

Conclusions

The study's goal was to characterise the efficacy and plant growth promoting activity of indigenous biocontrol bacterial isolates with broad antifungal activity in vitro. Furthermore, the selected biocontrol isolate will be tested in greenhouse conditions against *Fusarium oxysporum* on wheat crop. We believe that *Bacillus velezensis* (BA) could be useful as a biocontrol agent, liquid culture or efficient carrier material, and plant growth promoting inoculum in the production of agricultural crops.

Authors' Contributions

Investigation: SIA and SA; Conceptualization: HU and AK; Data curation: SIA; Funding acquisition: AK; Validation: BT and AR; Writing - original draft: NJ and SP; Writing - review and editing: NJ.
All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Ajayi-Oyetunde O, Bradley C (2018). *Rhizoctonia solani*: taxonomy, population biology and management of rhizoctonia seedling disease of soybean. Plant Pathology 67(1):3-17. *https://doi.org/10.1111/ppa.12733*

Amanda N (2015). Survey of *Fusarium oxysporum* f. sp. *vasinfectum* in the United States. The Journal of Cotton Science. The Cotton Foundation 19:328-336.

Awais M, Tariq M, Ali A, Ali Q, Khan A, Tabassum B, ... Husnain T (2017). Isolation, characterization and inter-relationship of phosphate solubilizing bacteria from the rhizosphere of sugarcane and rice. Biocatalysis and Agricultural Biotechnology 11:312-321. *https://doi.org/10.1016/j.bcab.2017.07.018*

Azeem S, Agha SI, Jamil N, Tabassum B, Ahmed S, Raheem A, ... Khan A (2022). Characterization and survival of broad-spectrum biocontrol agents against phytopathogenic fungi. Revista Argentina de Microbiología. *https://doi.org/10.1016/j.ram.2021.10.005*

Das A, Bhattacharya S, Murali L (2010). Production of cellulase from a thermophilic Bacillus sp. isolated from cow dung. American-Eurasian Journal of Agriculture and Environmental Sciences 8:685-691.

Dennis C, Webster J (1971). Antagonistic properties of species-groups of *Trichoderma*: III. Hyphal interaction. Transactions of the British Mycological Society 57(3):363-IN2. *https://doi.org/10.1016/s0007-1536(71)80077-3*

Dita M, Barquero M, Heck D, Mizubuti ES, Staver CP (2018). *Fusarium* wilt of banana: current knowledge on epidemiology and research needs toward sustainable disease management. Frontiers in plant science 9:1468. *https://doi.org/10.3389/fpls.2018.01468*

Eid AM, Fouda A, Abdel-Rahman MA, Salem SS, Elsaied A, Oelmüller R, ... Hassan SED (2021). Harnessing bacterial endophytes for promotion of plant growth and biotechnologi applications: an overview. Plants 10(5):935. *https://doi.org/10.3390/plants10050935*

Fernandes MFR, Ribeiro TG, Rouws JR, de Barros Soares LH, Zilli JÉ (2021). Biotechnological potential of bacteria from genera *Bacillus paraburkholderia* and *Pseudomonas* to control seed fungal pathogens. Brazilian Journal of Microbiology 1-10. *https://doi.org/10.1007/s42770-021-00448-9*

Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M (2009). Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine: Nanotechnology, Biology and Medicine 5(4):382-386. *https://doi.org/10.1016/j.nano.2009.06.005*

Ghodsalavi B, Ahmadzadeh M, Soleimani M, Madloo PB, Taghizad-Farid R (2013). Isolation and characterization of rhizobacteria and their effects on root extracts of *Valeriana officinalis*. Australian Journal of Crop Science 7(3):338-344.

Gilbert S, Xu J, Acosta K, Poulev A, Lebeis S, Lam E (2018). Bacterial production of indole related compounds reveals their role in association between duckweeds and endophytes. Frontiers in Chemistry 6:265. *https://doi.org/10.3389/fchem.2018.00265*

Haidar R, Roudet J, Bonnard O, Dufour MC, Corio-Costet MF, Fert M, ... Fermaud M (2016). Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. Microbiological Research 192:172-184. *https://doi.org/10.1016/j.micres.2016.07.003*

Jacob S, Sajjalaguddam RR, Kumar KVK, Varshney R, Sudini HK (2016). Assessing the prospects of *Streptomyces* sp. RP1A-12 in managing groundnut stem rot disease caused by *Sclerotium rolfsii* Sacc. Journal of General Plant Pathology 82(2):96-104. *https://doi.org/10.1007/s10327-016-0644-0*

Jasrotia S, Salgotra RK, Sharma M (2021). Efficacy of bioinoculants to control of bacterial and fungal diseases of rice (*Oryza sativa* L.) in northwestern Himalaya. Brazilian Journal of Microbiology:1-18. *https://doi.org/10.1007/s42770-021-00442-1*

Ji SH, Gururani MA, Chun SC (2014). Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiological Research 169:183-198. *https://doi.org/10.1016/j.micres.2013.06.003*

Jiang C-H, Yao X-F, Mi D-D, Li Z-J, Yang B-Y, Zheng Y, ... Guo J-H (2019). Comparative transcriptome analysis reveals the biocontrol mechanism of *Bacillus velezensis* F21 against *Fusarium wilt* on watermelon. Frontiers in Microbiology 10:652. *https://doi.org/10.3389/fmicb.2019.00652*

Joseph B, Ranjan Patra R, Lawrence R (2012). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). International Journal of Plant Production 1(2):141-152.

Kgatle M, Truter M, Ramusi T, Flett B, Aveling T (2018). *Alternaria alternata*, the causal agent of leaf blight of sunflower in South Africa. European Journal of Plant Pathology 151(3):677-688. *https://doi.org/10.1007/s10658-017-1402-7*

Khan A, Tabassum B, Aaliya K, Tariq M, Nasir I, Hassan S, ... Ponya Z (2019). The effectiveness of recombinant chitinase obtained from barley (*Hordeum vulgare* L.) against potato pathogens. Applied Ecology and Environmental Research 17(2):4147-4157. *https://doi.org/10.15666/aeer/1702_41474157*

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6):1547-1549.

Leiminger J, Bäßler E, Knappe C, Bahnweg G, Hausladen H (2015). Quantification of disease progression of *Alternaria* spp. on potato using real-time PCR. European Journal of Plant Pathology 141(2):295-309. *https://doi.org/10.1007/s10658-014-0542-2*

Lorck H (1948). Production of hydrocyanic acid by bacteria. Physiologia Plantarum 1(2):142-146. *https://doi.org/10.1111/j.1399-3054.1948.tb07118.x*

Lucero CT, Lorda GS, Anzuay MS, Ludueña LM, Taurian T (2021). Peanut endophytic phosphate solubilizing bacteria increase growth and p content of soybean and maize plants. Current Microbiology 78:1961-1972. *https://doi.org/10.1007/s00284-021-02469-x*

Madhaiyan M, Poonguzhali S, Kwon S-W, Sa T-M (2010). *Bacillus methylotrophicus* sp. *nov.*, a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. International Journal of Systematic and Evolutionary Microbiology 60(10):2490-2495. *https://doi.org/10.1099/ijs.0.015487-0*

Mitchell NJ, Bowers E, Hurburgh C, Wu F (2016). Potential economic losses to the US corn industry from aflatoxin contamination. Food Additives & Contaminants: Part A 33(3):540-550. *https://doi.org/10.1080/19440049.2016.1138545*

Nautiyal CS (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS microbiology Letters 170(1):265-270. *https://doi.org/10.1111/j.1574-6968.1999.tb13383.x*

Passari AK, Mishra VK, Leo VV, Gupta VK, Singh BP (2016). Phytohormone production endowed with antagonistic potential and plant growth promoting abilities of culturable endophytic bacteria isolated from *Clerodendrum colebrookianum* Walp. Microbiological Research 193:57-73. *https://doi.org/10.1016/j.micres.2016.09.006*

Pastuszak J, Szczerba A, Dziurka M, Hornyák M, Kopeć P, Szklarczyk M, Płażek A (2021). Physiological and biochemical response to *Fusarium culmorum* infection in three durum wheat genotypes at seedling and full anthesis stage. International Journal of Molecular Sciences 22(14):7433. *https://doi.org/10.3390/ ijms22147433*

Pereira BdA, Castro-Silva MA (2010). Rizobactérias formadoras de endósporos associadas à *Tibouchina urvilleana* de áreas impactadas por rejeitos da mineração do carvão. Revista Brasileira de Ciência do Solo 34(2):563-567. https://doi.org/10.1590/s0100-06832010000200030

Ramzan M, Tabassum B, Nasir IA, Khan A, Tariq M, Awan MF, ... Toufiq N (2016). Identification and application of biocontrol agents against cotton leaf curl virus disease in *Gossypium hirsutum* under greenhouse conditions. Biotechnology and Biotechnological Equipment 30(3):469-478. https://doi.org/10.1080/13102818.2016.1148634

Rana A, Sahgal M, Johri B (2017). *Fusarium oxysporum*: genomics, diversity and plant–host interaction. Developments in Fungal Biology and Applied Mycology. Springer, pp 159-199 https://doi.org/10.1007/978-981-10-4768-8_10

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4):406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Schwyn B, Neilands J (1987). Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160(1):47-56. https://doi.org/10.1016/0003-2697(87)90612-9

Shafi U, Mumtaz R, Shafaq Z, Zaidi SMH, Kaifi MO, Mahmood Z, Zaidi SAR (2022). Wheat rust disease detection techniques: a technical perspective. Journal of Plant Diseases and Protection 1-16. https://doi.org/10.1007/s41348-022-00575-x

Shimodaira H, Hasegawa M (2001). CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17(12):1246-1247. https://doi.org/10.1093/bioinformatics/17.12.1246

Sivasankari B (2016). Indole-3-Acetic acid production by the bacterial strains isolated from vermicomposts in the presence and absence of tryptophan. International Journal of Innovative Research in Science, Engineering and Technology 5(5):92-107.

Syed R, Lodhi A, Jiskani M, Rajput K, Khaskheli M, Khanzada M, ... Rajput A (2015). First report of Panama wilt disease of banana caused by *Fusarium oxysporum* f. sp. *cubense* in Pakistan. Journal of Plant Pathology 97(1):213.

Tabli N, Rai A, Bensidhoum L, Palmieri G, Gogliettino M, Cocca E, ... Nabti E (2018). Plant growth promoting and inducible antifungal activities of irrigation well water-bacteria. Biological Control 117:78-86. https://doi.org/10.1016/j.biocontrol.2017.10.010

Tewoldemedhin Y, Lamprecht S, McLeod A, Mazzola M (2006). Characterization of *Rhizoctonia* spp. recovered from crop plants used in rotational cropping systems in the Western Cape province of South Africa. Plant Disease 90(11):1399-1406. https://doi.org/10.1094/PD-90-1399

Toppo S, Naik U (2015). Isolation and characterization of bacterial antagonist to plant pathogenic fungi (*Fusarium* spp.) from agro based area of Bilaspur. International Journal of Research Studies in Biosciences 6-14.

Tsegaye Z, Gizaw B, Tefera G, Feleke A, Chaniyalew S, Alemu T, Assefa F (2019). Isolation and biochemical characterization of plant growth promoting (pgp) bacteria colonizing the rhizosphere of Tef crop during the seedling stage. Biomedical Journal of Scientific and Technical Research 14:013-027.

Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, ... Otani H (2013). Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. FEMS Microbiology Reviews 37(1):44-66. https://doi.org/10.1111/j.1574-6976.2012.00350.x

Vasebi Y, Safaie N, Alizadeh A (2013). Biological control of soybean charcoal root rot disease using bacterial and fungal antagonists In Vitro and greenhouse condition. Journal of Crop Protection 2(2):139-150. https://doi.org/10.1515/jppr-2016-0004

Velivelli SL, Lojan P, Cranenbrouck S, de Boulois HD, Suarez JP, Declerck S, ... Prestwich BD (2015). The induction of Ethylene response factor 3 (ERF3) in potato as a result of co-inoculation with *Pseudomonas* sp. R41805 and *Rhizophagus irregularis* MUCL 41833–a possible role in plant defense. Plant Signaling & Behavior 10(2):e988076. https://doi.org/10.4161/15592324.2014.988076

Vipul V, Alpika V, Akhilesh K (2012). Isolation & production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India. Advances in Applied Science Research 3(1):171-174.

Wang C, Zhao D, Qi G, Mao Z, Hu X, Du B, ... Ding Y (2020a). Effects of *Bacillus velezensis* FKM10 for promoting the growth of *Malus hupehensis* Rehd. and inhibiting *Fusarium verticillioides*. Frontiers in Microbiology 10:2889. https://doi.org/10.3389/fmicb.2019.02889

Wang S, Sun L, Zhang W, Chi F, Hao X, Bian J, Li Y (2020b). *Bacillus velezensis* BM21, a potential and efficient biocontrol agent in control of corn stalk rot caused by *Fusarium graminearum*. Egyptian Journal of Biological Pest Control 30(1):9. https://doi.org/10.1186/s41938-020-0209-6

Yang C, Hamel C, Vujanovic V, Gan Y (2011). Fungicide: modes of action and possible impact on nontarget microorganisms. ISRN Ecology 2011:130289. *https://doi.org/10.5402/2011/130289*

Yigit F, Dikilitas M (2007). Control of fusarium wilt of tomato by combination of fluorescent *Pseudomonas*, non-pathogen *Fusarium* and *Trichoderma harzianum* T-22 in greenhouse conditions. Plant Pathology Journal 6:159-163. *https://doi.org/10.3923/ppj.2007.159.163*

Yoon M-Y, Cha B, Kim J-C (2013). Recent trends in studies on botanical fungicides in agriculture. The Plant Pathology Journal 29(1):1-9. *https://doi.org/10.5423/ppj.rw.05.2012.0072*

Zhu L, Ni W, Liu S, Cai B, Xing H, Wang S (2017). Transcriptomics analysis of apple leaves in response to *Alternaria alternata* apple pathotype infection. Frontiers in Plant Science 8:22. *https:// doi.org/10.3389/fpls.2017.00022*