



## Isolation and characterization of bacteria from two soil samples and their effect on wheat (*Triticum aestivum* L.) growth promotion

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**ABSTRACT:** Since ancient times, soil bacteria play an important role on crop growth and yield by genetic transformation naturally. But the continuous use of chemical fertilizers reduces their number and proper environment for multiplication. Seed treatment with beneficial bacteria provides nutrients for the growth of crop plants. Thus, soil bacteria were isolated, their growth characteristics and effect on wheat growth were observed. The maximum growth of Isolate A and Isolate B was observed at pH 5.5, 7.0 and 33°C, 35°C respectively. Morphological characteristics indicated that Isolate A and Isolate B were gram-positive. But both bacteria were non-motile. In Biochemical test, both of them showed positive result in the methyl red test, catalase test, urea test, starch hydrolysis test, and negative in TSI (Triple Sugar Iron) test, mannitol salt test. Isolate B showed positive result in BSA, MacConkey test and EMB (Eosin Methylene Blue) test and Isolate A showed negative result in BSA (Bismuth Sulphite Agar), MacConkey test and EMB test. Besides, both of the bacteria were multi-drug resistance showing resistance to penicillin, amoxicillin, ampicillin cefuroxime, and ceftazidime. 16S rRNA gene sequencing identified the isolate A and Isolate B as *Bacillus thuringiensis* and *Bacillus anthracis*. After 6 hours of wheat seed treatment germination percentage, fresh root and shoot weight, root and shoot dry weight, relative water content of both root and shoot, and plant growth was enhanced by *Bacillus thuringiensis* and *Bacillus anthracis*. *Bacillus anthracis* was more capable than *Bacillus thuringiensis* for increasing germination rates, both root and shoot growth of wheat. It indicated that *Bacillus anthracis* and *Bacillus thuringiensis* mediated growth improvement of wheat is possibly originated in roots.

**KEYWORDS:** Soil microbes, Characterization, Wheat, Growth parameters.

### INTRODUCTION

Soil microorganisms can fix nitrogen, multiply, and release oxygen into the atmosphere and affect soil structure and fertility [1, 2, 3]. These microbes have different characteristics and their advantageous function in soil. Soil bacteria have been used for decades for crop production [4]. Nowadays, in an integrated plant nutrient management system, microbiological approaches have become more popular for crop improvement and yield rather than chemical fertilizers. Use of plant growth promoting rhizobacteria (PGPR) has played a crucial role in crop production, in particular, developing

sustainable systems in the plant ecosystem [5, 6]. Symbiotic and non-symbiotic bacteria are now being used worldwide for the enhancement of plant productivity [7, 8]. Besides this, non-symbiotic nitrogen-fixing *Bacillus* sp. is also being used to inoculate large areas of cultivable land around the world for enhancing plant productivity [9]. *Bacillus* and *Paenibacillus* are phosphate-solubilizing bacteria and were applied to soils for enhancing the phosphorus status of plants [10]. Phosphorus is essential for the vigor of all plants and processes from the beginning of seedling growth. *Bacillus* bacterial species perform many important ecosystem services in the soil including improved soil

structure and soil aggregation, recycling of soil nutrients, and water recycling. PGPR have been playing a progressive role in the development of sustainable agricultural systems [11]. Generally, PGPR function in three different ways: particular compounds that are synthesized for the plants [12, 13], facilitating the uptake of some crucial nutrients from the soil [14, 15], and prohibiting the plants from diseases [16-19]. The PGPR mediated plant growth and yield improvement of many crops are not fully understood [20]. Phosphorus (P), an important macronutrient plays a crucial role in plant growth and development [21]. Organic and inorganic phosphates are found in soils as macronutrients. Both organic and inorganic phosphorus induce PGPR for increasing plant yields [22, 23]. Some reports showed microbial phosphorus release from organic P sources [24]. Bacteria strains such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, and *Erwinia* can solubilize insoluble inorganic phosphate compounds [25]. But *Pseudomonas*, *Bacillus*, and *Rhizobium* are the most powerful phosphate solubilizers [26, 27]. *Bacillus* bacterial genera holds potential for developing biofertilizer and biocontrol agents and continued research with these genera will make *Bacillus* as a potential PGPR and reveal a new era of achieving sustainable crop yield in agriculture. Thus, the present study was designed to isolate and characterize of *Bacillus* spp. from flooded and unflooded soil and to observe their effect on wheat seeds for plant-growth promoting morphological traits.

## MATERIALS AND METHODS

### Sample collection

Two soil samples were collected from the different land (Flooded and unflooded) of Bogura district. One of the lands is flooded every year but the other land is never flooded. Samples were aseptically collected in sterile plastic container and transported to the Microbiology Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, Bangladesh. Then samples were stored in ice for 16 hours until subsequent analysis in the laboratory. The wheat (BARI gom-33) was collected from regional wheat and maize research center, Shyampur, Rajshahi.

### Chemicals

Peptone, yeast extract, bacteriological agar, sodium chloride, ethanol, methanol, sodium hydroxide (NaOH), hydrochloric acid (HCl), crystal violet, and grams iodine were obtained from bioWORLD, USA. Basal salt, mannitol salt, macConkey agar, urea agar, starch agar, TSI agar, simmons citrate agar, EMB agar were purchased from Merck, Germany. All other chemicals and solvents were in analytical grade.

### Preparation of mixed bacterial culture

Two (2) gm of each soil sample was mixed with 100 ml distilled water in a beaker and filtered through the whatman filter paper. After filtration 100µl solution was added in Luria-Bertani (LB) liquid medium and incubated for 16-18 hours at 37°C temperature to prepare bacterial mixed culture.

### Isolation of pure bacterial culture

After serial dilution, from each tube 100µl of diluted samples were transferred into nutrient agar plates and incubated at 37°C for 24hours. Then single colony was selected and streaked several times for pure bacterial colony. Pure single colony was transferred into LB liquid medium for store and further use.

### Morphological and biochemical test

Morphological and biochemical tests were used for specific identification of bacteria. Isolated bacteria were characterized by several morphological and biochemical tests such as gram staining, motility, catalase, methyl red, MacConkey, Mannitol, Urea Hydrolysis, Starch Hydrolysis, Triple Sugar Iron (TSI), Citrate, Bismuth Sulfite Agar (BSA), and Eosin Methylene Blue (EMB) Agar test.

### Role of pH and temperature on bacterial growth

To observe the effect of pH on bacterial growth, the culture medium was adjusted to pH ranging from 3.0 to 8.0 with 0.5 intervals. For temperature effect data were recorded at 25°C, 30°C, 33°C, 35°C, 40°C and 45°C. Bacterial cell density was determined by measuring

optical density at 600 nm with a UV-Vis spectrophotometer (Analytic Jena, Germany).

### Antibiotic sensitivity test of isolated bacteria

Different antibiotics like Penicillin, Amoxicillin, Gentamycin, Tetracycline, Ciprofloxacin, Cefuroxime, Cefixime, Ampicillin, Erythromycin, Erythromycin, Kanamycin, Ceftazidime, and Doxycycline were used for antibiotic sensitivity test. Antibiotic discs were placed carefully on the respective plates and incubated overnight at 37°C. After overnight incubation the zone was observed on the plate and measured with the help of mm scale. Gentamycin was used as a control.

### Molecular methods for species identification

The 16S rRNA gene was sequenced from Invent technology and compared with other sequences from the gene bank database using Basic Local Alignment Search Tool (BLAST) available from the website [www.ncbi.nlm.nih.gov/Blast](http://www.ncbi.nlm.nih.gov/Blast) to identify bacteria [28].

### Seed treatment

Fresh bacterial culture was prepared 1 day before seeds treatment. Then seeds were washed thoroughly and immersed in distilled water for 30 min. After immersion they were sterilized with 70% (v/v) ethanol for 3 min and properly washed with distilled water. Then seeds were transferred into fresh bacteria culture and were shook in the shaker for 0.5 h, 1h, 2h, 4h, 6h, 8h and 10h at 37°C. After shaking 30 seeds were placed into each 90 mm petri dish (with three replications) which containing 2 layers moistened of tissue papers at the bottom for germination. The petri dishes were taken on room temperature for germination. The treated and control seeds were watered every day for germination.

### Germination percentage analysis

The germination percentage was recorded after 7 days of treatment. The germination percentage was calculated by using the following equation:

Germination percentage = (Number of seeds germinated / total number of seeds inoculated) \* 100

### Plant growth analysis

Plant growth was measured after 7 days of planting in petri dishes. Root and shoot length were measured in cm and number of roots were counted and compared with the control.

### Determination of shoot and root dry weight

To determine shoot and root dry weight, 10 days germinated plants were harvested from the petri dish. Roots and shoots were separated from each other. Roots were washed with distilled water to remove the adherent tissue paper and kept in the dryer incubator at 70°C for 3 days, the dry weight of shoot and root were measured by using electrical balance.

### Relative water content (RWC) in root and shoot

The relative water content (RWC) of both roots and shoots for each treatment was calculated according to the formula of Weatherly [29].

$$RWC = [(FW-DW)/(TW-DW)] \times 100.$$

Where, FW= Fresh weight of shoot/root

DW= Dry weight of shoot/root

TW= Turgid weight of shoot/root

### Statistical analysis

All experiments were carried out in triplicates and the results are presented as the mean of three independent observations. Significance of each group data was analyzed statistically at  $P \leq 0.05$  by ANOVA one-way followed by Duncan's Multiple Range Test (DMRT) in SPSS Statistics 20 software. Graphs were prepared using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

## RESULTS

### Pure bacterial colony isolation

Isolate A (flooded) and Isolate B (unflooded) were isolated from mixed bacterial culture by using the streak plate method and serial dilution (Fig.1). Colonies were selected according to their morphological characters and nature.



**Figure 1.** Streaking and pure LB agar plate of Isolate A and Isolate B bacterial strain.

### Morphological and biochemical characteristics

The morphological characteristics of isolated bacteria are shown in Fig. 1 and Table 1. Both Isolates were almost rod-shaped, gram-positive, and yellowish. Biochemical test results are also shown in Table 1 which indicated that the Isolate-A was positive for Methyl Red test, Catalase test, Urea Hydrolysis test, Starch Hydrolysis test and negative for Motility test, MacConkey test, Mannitol salt test, Simmons' Citrate test, Bismuth Sulfate Agar (BSA) test, Eosin Methylene Blue (EMB) agar test. Besides this, Isolate B was positive for Methyl Red test, Catalase test, MacConkey test, Urea Hydrolysis test, Starch Hydrolysis test, Simmons' Citrate test and negative for Motility test and Mannitol salt test.

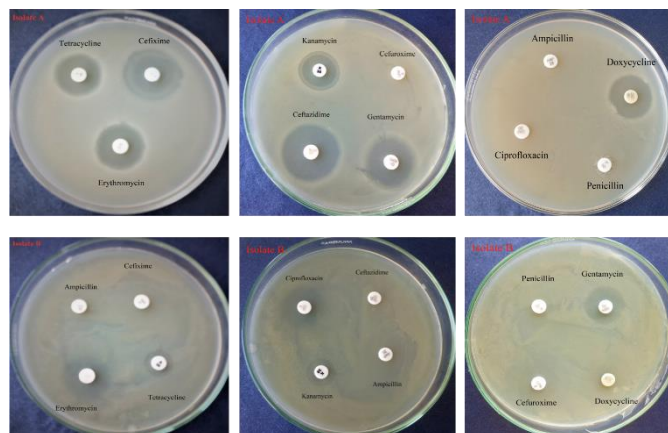
**Table 1.** The results of morphological and biochemical test of Isolate A and Isolate B.

No.	Test name	Results	
		Isolate A	Isolate B
01.	Gram staining test	Gram positive	Gram positive
02.	Motility test	-	-
03.	Methyl Red test	+	+
04.	Catalase test	+	+
05.	MacConkey test	-	-
06.	Mannitol salt test	-	-
07.	Urea Hydrolysis test	+	+
08.	Starch Hydrolysis test	+	+
09.	Triple Sugar Iron (TSI) test	H <sub>2</sub> S-, gas-	H <sub>2</sub> S+, gas-
10.	Simmons' Citrate test	-	+
11.	Bismuth Sulfite Agar (BSA) test	-	+
12.	Eosin Methylene Blue (EMB) agar test	-	+

### Antibiotic sensitivity test

The results (Fig. 2) showed that, Isolate A had no intermediate resistance but was susceptible to gentamycin, tetracycline, ciprofloxacin, erythromycin, kanamycin, and doxycycline and resistant to penicillin,

ampicillin, amoxicillin, cefuroxime, cefixime and ceftazidime (Table 2). On the other hand, Isolate B was intermediate resistant to tetracycline and susceptible to gentamycin, ciprofloxacin, cefixime, erythromycin, kanamycin, doxycycline and resistant to penicillin, amoxicillin, ampicillin, cefuroxime, and ceftazidime. So, Isolate A and isolate B did not show similar characteristics in antibiotic sensitivity tests.



**Figure 2.** Antibiotic sensitivity test result of Isolate A and isolate B

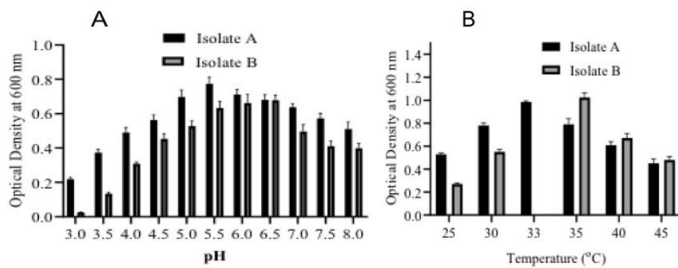
**Table 2.** Antibiotic sensitivity test for detection of the resistance pattern of isolated bacteria

Names of antibiotics	Zone of inhibition (Mean ±SD)			
	Isolate A (mm)	Resistant pattern	Isolate B (mm)	Resistant pattern
Penicillin	6.50±0.50	Resistant	6.17±0.29	Resistant
Amoxicillin	7.00±0.50	Resistant	6.33±0.58	Resistant
Gentamycin	25.02±0.53	Susceptible	25.83±0.29	Susceptible
Tetracycline	22.00±0.50	Susceptible	10.50±0.50	intermediate
Ciprofloxacin	26.00±0.87	Susceptible	21.0±0.50	Susceptible
Cefuroxime	6.83±0.76	Resistant	6.67±0.76	Resistant
Cefixime	29.17±0.76	Susceptible	20.50±0.50	Susceptible
Ampicillin	6.67±0.76	Resistant	6.67±0.76	Resistant
Erythromycin	28.83±1.04	Susceptible	17.50±1.00	Susceptible
Kanamycin	19.17±0.29	Susceptible	15.00±0.87	Susceptible
Ceftazidime	6.50±0.87	Resistant	6.167±0.29	Resistant
Doxycycline	22.50±0.50	Susceptible	21.50±0.50	Susceptible

Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm

### Role of pH and temperature on bacterial growth

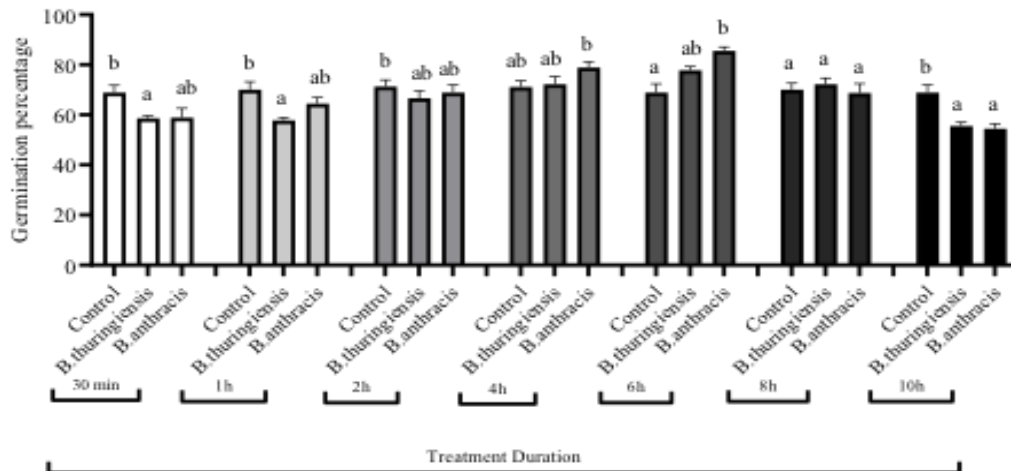
The optimal growth conditions of Isolate A and Isolate B were determined with different pH ranging from 3.0 to 8.0. Isolate A and Isolate B showed maximum growth at pH 5.5 and 6.5 respectively (Fig. 3A). The temperature effect on bacterial growth was also measured in various temperatures ranging from 25 to 45°C with an interval of 5.0. Isolate A and Isolate B showed their maximum growth at 33°C and 35°C respectively (Fig. 3B).



**Figure 3.** Effect of pH and temperature on the growth of Isolate A and Isolate B bacteria after 24 hours of incubation. Error bars presented mean  $\pm$  standard deviation of triplicates of three independent experiments.

## Morphological and physiological characteristics of wheat seedlings

*Bacillus thuringiensis* and *Bacillus anthracis* treatment caused a remarkable increase in root length, shoot length, both root and shoot dry weight in wheat seedlings compared to controls. Significant changes were observed in shoot length, root length, fresh shoot weight and fresh root weight in wheat seedlings compared to controls due to seed treatment with the *B. anthracis* but in case of *B. thuringiensis* non-significant changes were



**Figure 4.** Germination percentage of both treated and untreated wheat seeds in different time duration. Different letters indicate significant differences between mean  $\pm$ SD of treatments (n=3) at  $p < 0.05$  significance level.

## Species identification

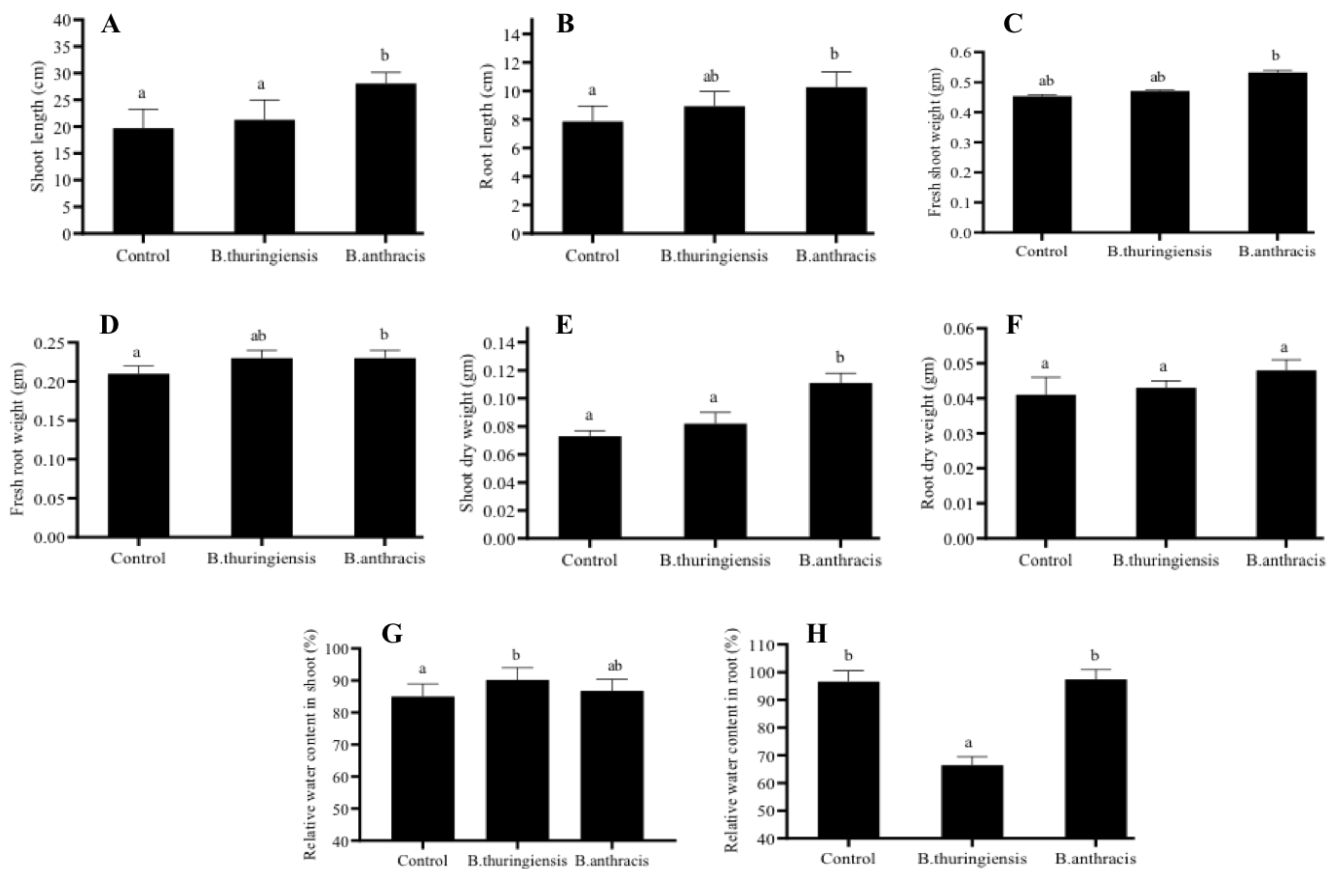
From the 16S rRNA gene sequence comparison, Isolate A showed 99.51% similarity with *Bacillus thuringiensis* and Isolate B showed 99.04% similarity with *Bacillus anthracis*. Thus, Isolate A and Isolate B were confirmed as *Bacillus thuringiensis* and *Bacillus anthracis* respectively.

## Germination percentage

Among different treatment duration, seed germination percentage was increased due to treatment for 6 hours (Fig.4) with both the bacterial strains in comparison to control (Fig.4). After 7 days, *Bacillus thuringiensis* treated seed germination rate was 77.78% whereas *Bacillus anthracis* treated seed germination rate was 85.56%. Between these two bacteria, *Bacillus anthracis* showed better results in seed germination (Fig.4)

observed in the above mentioned characters (Fig.5)

However, shoot dry weight significantly increased in seedlings treated with *B. anthracis*. Whilst other characteristics such as root dry weight, relative water content in shoot and root showed no significant changes due to treatment with both the bacteria, but relative water content in root was remarkably decreased due to the treatment with *B. thuringiensis* (Fig.5). In all the parameters, *Bacillus anthracis* showed more significant results than control and *Bacillus thuringiensis*.



**Figure 5.** Morphological characteristics of wheat seedlings treated with *Bacillus thuringiensis* and *Bacillus anthracis*. Shoot length of wheat seedlings (A), Root length of wheat seedlings (B), Fresh shoot weight (C), Fresh root weight (D), Shoot dry weight (E), Root dry weight (F), Relative water content in shoot (G) and Relative water content in root (H). Different letters indicate significant differences between mean  $\pm$ SD of treatments (n=3) at  $p < 0.05$  significance level.

## DISCUSSION

Improvement of crop plants is highly desirable to fulfill the demand of the vast population. Beneficial bacterial treatment of seeds has been proven to be efficient in crops species whilst induced mechanism of PGPR for germination and growth remains to be uncertain. This study reveals new insights into the role of seed treatment technology for triggering the germination and growth of wheat plants. Application of beneficial bacteria for seed treatment to increase yield and reduce use of pesticide and chemical fertilizer, which is harmful to both humans and environments. So, it is very attractive, and benefits could be considerable. The commercial use of beneficial bacteria as a common agricultural practice will depend on such aspects as cost-benefit ratios, wide-spread applicability of specific strains. *Bacillus thuringiensis* and *Bacillus anthracis* preferred ecological niche is also home to various other

types of soil micro-organisms due to its rich nutrient availability.

In this study we also optimized and characterized the primarily isolated strains [30]. They showed maximal growth at different pH and temperature. However, isolate-A revealed maximum growth at pH 5.5 in 33°C and isolate B at pH 6.5 in 35°C after 24 hours incubation. Many bacteria are resistant to some of common antibiotics and these antibiotics can't kill the bacteria. So, antibiotics sensitivity analysis is a useful tool to help quickly determine if bacteria are resistant to certain drugs. Examples of antibiotic-resistant infections include: a persistent sore throat, a recurring urinary tract infection (UTI) and an unresponsive case of pneumonia [31]. Isolate A was susceptible to gentamycin, tetracycline, ciprofloxacin, erythromycin, kanamycin, and doxycycline and resistant to penicillin, ampicillin, amoxicillin, cefuroxime, cefixime and ceftazidime. On the other hand, Isolate B was intermediate resistant to tetracycline and susceptible to gentamycin,

ciprofloxacin, cefixime, erythromycin, kanamycin, doxycycline and resistant to penicillin, amoxicillin, ampicillin, cefuroxime, and ceftazidime

After isolation, two *Bacillus* spp. were used for seed treatment and their effect on seed germination, root-shoot growth, fresh root-shoot weight, root-shoot dry weight, relative water content of root-shoot of wheat were measured. After 7 days, control wheat variety showed 68.89% germination with an average of 19.73 cm shoot and 7.87 cm root length whereas seeds treated with *Bacillus thuringiensis* showed maximum 77.78% germination with 3.4 cm shoot and 8.93 cm root length but 85.56% germination with 28.07 cm shoot and 10.27 cm length was achieved by *Bacillus anthracis* seed treatment. Plant growth promoting rhizobacteria (PGPR) can improve the extent of plant growth directly or indirectly. A study reported that *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Bacillus*, and *Serratia* could increase plant growth [32]. Both bacteria have the ability to increase the growth of wheat plant but overall *Bacillus anthracis* showed the best results compared to *Bacillus thuringiensis* on root-shoot growth. *Bacillus megaterium* strain (RmBm31) that possesses a wide range of genomic features linked to plant growth promotion. [33]. It acts as a PGPR with biological promotion of different characteristics of plant growth [34]. Many *Bacillus* species are well-known plant-growth promoters, capable of promoting plant nutrient uptake, controlling phytopathogens, and producing phytohormones [35]. All these enhanced the plant growth as a result of their ability to fix nitrogen. Other mechanism may be attributed to growth promotion by plant growth promoting hormones production and other PGR activities [36].

The RWC showed 86.83% in shoot and 97.38% in root treated with *Bacillus anthracis*, whereas seeds treated with *Bacillus thuringiensis* showed a maximum of 90.26% in shoot and 66.43% in root RWC but control showed 85.04% in shoot and 96.63% in root RWC. In this case, seed treated with *Bacillus anthracis* increased both root and shoot relative water content. Water status in leaf is related to several leaf physiological variables, such as leaf turgor, growth, stomatal conductance, photosynthesis and respiration [37]. Water content is used to quantify the water presence in shoot and root tissues. So, shoot and root water content is a useful

indicator of plant water balance. Potential water provides energetic status of shoot and root [38].

Though *Bacillus anthracis* is considered as an obligate agent that cause anthrax in humans, livestock and wildlife, it also may promote plant growth when inoculated into carcass site soil [39]. *Bacillus anthracis* can interact with plants (*Enneapogon desvauxii*) and promote anthrax transmission [39]. So, for considering this bacterium as wheat growth promoting purpose, more studies should be carried out about its transmission through this crop and subsequent effects on yield and other quantitative traits of wheat (Fig. 6).

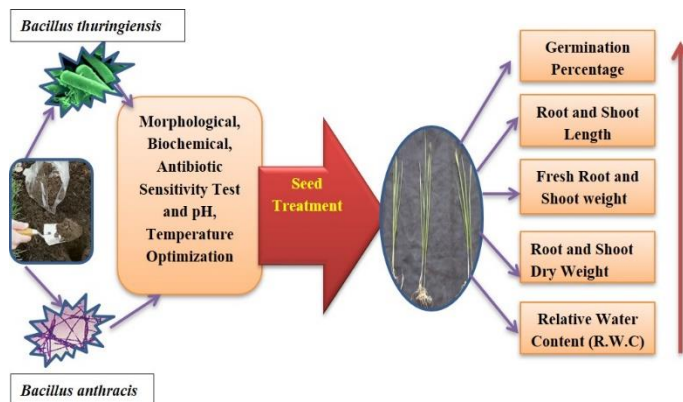


Figure 6. Schematic diagram of summary.

## CONCLUSION

Our findings collectively point out that inoculation of wheat seed with both *Bacillus thuringiensis* and *Bacillus anthracis* for 6 hours could be the way for rapid enhancement of plant morphological characteristics. Thus, both the bacteria can be used for wheat growth promotion at the field level.

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## AUTHOR CONTRIBUTIONS

GKP and MAS conceived the idea. GKP performed all experiments. GKP and SM prepared manuscript. MAS supervised the research and revised the manuscript. SZ,

MSU and MAS arranged the whole facilities for the research. KN, TJ, MLM and MNH helped for performing the experiments. All of the authors read and approved the manuscript.

## CONFLICTS OF INTEREST

The author (s) declared no potential conflicts of interest with respect to the research, authorship and publication of this article.

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