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Bacillus- aided boron nutrition improves productivity of bread wheat (*Triticum aestivum* L.)

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Abstract

Boron (B) deficiency can significantly impact wheat (*Triticum aestivum* L.) grain yield. Although B is present in soil upto 200 ppm but only 5% of it is available for the plants while the rest is in non-available form. However, some plant growth promoting rhizobacteria (PGPR), termed as B solubilizing bacteria, has the potential to augment B availability in the rhizosphere. Therefore, the present study was designed to investigate the effect of B application, with and without the use of B solubilizing bacteria (*Bacillus* MN54), on B availability, wheat performance and grain B concentration in two wheat cultivars (Faisalabad-2008 and Lasani-2008). Boron was applied to wheat through seed priming (0.01 *M* B), seed coating (250 mg kg^{-1} seed), soil application (1.00 kg ha^{-1}) and foliar spray (0.01 *M* B), with hydropriming as control treatment. The results revealed that plants treated with *Bacillus* MN54 showed higher concentrations of organic acids in their root exudates, leading to improved wheat yield and grain B concentration. Furthermore, the combined application of B and *Bacillus* MN54 by various methods further improved the efficacy of the inoculant. Specifically, B and *Bacillus* MN54 application as seed priming resulted in the highest levels of organic acids in root exudates and grain yield (27%) followed by soil B + *Bacillus* MN54 application (25%) compared to control. Notably, the application of B and *Bacillus* MN54 as soil (21%) and foliar (23%) treatments yielded the highest grain B concentration. In conclusion, seed priming with B in combination with *Bacillus* MN54 has the potential to simultaneously improve wheat grain yield and grain B concentration.

Keywords: *Bacillus*, Grain yield, Nutrient uptake, Organic acids, Plant growth promoting rhizobacteria

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Introduction

Wheat (*Triticum aestivum*) is a crucial staple crop, providing essential nutrition to populations worldwide (Peña-Bautista et al., 2017; Iqbal et al., 2018; Taglieri et al., 2021). It is considered an important and nutritionally complete food source, supporting human health (Abbas & Shafique, 2019; Singh et al., 2019). Unlike other cereals, wheat offers a wide range of nutrients in different food forms, making it a cornerstone of global nutrition (Soomro et al., 2009; Mehmood et al., 2020; Alamgeer et al., 2022; Dinsa & Balcha, 2024). In Pakistan, wheat plays a vital role in the agricultural sector, contributing 9.0% to agriculture and 2.2% to the country's GDP (Pakistan Economic Survey, 2023-24). With over 9.0 million hectares under cultivation and an average yield of 3200 kg ha^{-1}, wheat is an essential crop in Pakistan's economic and agricultural landscape (Pakistan Economic Survey, 2023-24). However, wheat yield often fall short of their potential due to factors such as the unavailability of recommended seed varieties, planting delays, improper cultivation methods, fertilizer imbalances and inefficient water management (Khan et al., 2007; Zawar et al., 2024).

For optimal growth, development, and yield, plants require a balanced supply of essential nutrients (Baig et al., 2018; Mehmood et al., 2022; Azam et al., 2023). A proper mix of primary nutrients nitrogen (N), phosphorous (P), potassium (K), secondary nutrients; sulfar (S) and certain micronutrients like zinc (Zn) and boron (B) is necessary to boost wheat production (Watanabe et al., 2007; Ahmad & Aslam, 2018). Although micronutrients are required in small amounts, their adequate supply plays a crucial role in enhancing yield, improving cell physiology, and positively influencing plant performance (Dimkpa and Bindraban, 2016; Yousaf et al., 2020; Jamilah et al., 2024). Boron is a critical micronutrient for plants, and its deficiency can impair plant development and function (Vera-Maldonado et al., 2024). Boron contributes to cell wall biosynthesis and structural integrity (Shireen et al., 2018; Pereira et al., 2021) by forming borate esters with rhamnogalacturonan (RG-II), which enhance the porosity and flexibility of the cell wall (Funakawa and Miwa, 2015; Nejad and Etesami, 2020). In the roots of *Arabidopsis thaliana*, B is vital for crosslinking RG-II and assembling pectin (Camacho-Cristóbal et al., 2008). Furthermore, B plays a role in stimulating reproductive tissues, enhancing seed quality, regulating ion transport across membranes, promoting cell division and elongation, supporting protein cytoskeletal function, and assisting in antioxidant metabolism, sugar transport, oxidoreductase activity and the synthesis and transport of plant hormones (Lu et al., 2015; Shireen et al., 2018).

Boron deficiency in wheat results in empty and shriveled anthers, non-viable pollens, pollen tube rupture, dropping of floral buds and failure of grain set that affects the reproductive performance of plants (Farhan et al., 2021). To compensate for B deficiency in plants, B-containing fertilizers are commonly employed through various methods, including soil application, foliar spraying and seed treatment (seed priming and coating) (Moradi and Siosemardeh, 2023). Among these methods, soil and foliar applications are widely used, each with some merits and demerits. Soil application can lead to potential nutrient loss through immobilization and leaching, with leached nutrients accumulating in the soil to potentially toxic levels. Conversely, foliar application can cause leaf burns if dosage is not pre-optimized (Yin, 2024). Nonetheless applying nutrients through seed treatment not only results in higher yield and improved plant growth compared to soil and foliar application methods but is also a cost-effective method (John et al., 2022).

Similar to other nutrients, the B deposits present in soil are in the range of 2 to 200 ppm but only 5% of this is available to the plants due to various soil related factors (Patgiri et al., 2023). Soil microbes play a crucial role in influencing soil's micronutrient availability to plants through various processes, including chelation, solubilization and secretion of root exudates (Adomako et al., 2023). Specifically, PGPR contributes to enhancing the availability of essential elements to wheat by releasing organic acids within the rhizosphere that aid in nutrient dissolution and absorption (Thepbandit & Athinuwat, 2024). Plant growth promoting rhizobacteria further stimulate plant growth through mechanisms that involve improved nutrient availability, N fixation, enzyme synthesis, siderophore production and synthesis of growth hormones (Singh et al., 2022). The process of mineralization and release of B in the rhizosphere occurs at a relatively slower rate. Usable B in soil is tightly held by soil organic matter, and the sole method for releasing this tightly held B and making it accessible to plant uptake is the lowering of rhizosphere soil pH. This pH adjustment is typically achieved by soil microorganisms that efficiently break down organic matter and release organic acids (Patgiri et al., 2023). The acidic pH environment is conducive to converting otherwise inaccessible forms of B into readily available forms (Arrobas et al., 2023). Numerous studies have documented the enhanced availability of micronutrients facilitated by rhizosphere microbes (Tsegaye et al., 2022).

While various methods of B application have demonstrated their effectiveness in enhancing wheat performance in field conditions, a relatively unexplored avenue involves the concurrent use of B in combination with B solubilizing PGPR. Addressing this gap, our study investigated the synergy between *Bacillus* MN54 and B, assessing their impact on wheat productivity and yield under organic matter deficient calcareous soils. The study further explored the interaction of *Bacillus* MN54 and B application methods on rhizosphere soil organic acid production and B mineral concentration in grains of wheat.

Materials and Methods

Experimental site and treatments

The experiment was conducted in a glass house at University of Agriculture, Faisalabad (latitude 31.7°N, longitude 73.98°E), Pakistan, for two consecutive winter seasons. The cultivars, Faisalabad-2008 and Lasani-2008, used in this study were collected from the Wheat Research Institute, Faisalabad, Pakistan. B as borax ($Na_2B_4O_7.10H_2O$) was applied as seed priming (0.01 *M*), seed coating (250 mg kg^{-1} seed), foliar application (0.01 *M*), soil application (1 kg ha^{-1}) with or without *Bacillus* MN54 whereas hydropriming was taken as control treatment. Experimental treatments were organized following a Completely Randomized Design (CRD) in a factorial setting. During 1st year, the trial was sown with four replications (for statistical reliability and minimizing experimental error) whereas during 2nd year, the trial was sown using eight replications; 4 repeats were used for destructive sampling while 4 repeats were used to record yield and yield components.

The bacterial strain *Bacillus* MN54 was originally isolated from maize rhizosphere (Ali et al., 2021) and the performance of strain *Bacillus* MN54 was also checked in various experiments for improving the development and yield of a

number of crops in standard and anxious environments (Samreen et al., 2019). Orbital shaking incubator (Firstek Scientific, Tokyo, Japan) was employed to prepare the inoculum of strain *Bacillus* MN54 at 180 rev min^{-1} and 28°C for 48 h (Suja et al., 2014). The absorbance of the culture at 600 nm was recorded using a spectrophotometer (UV-4000, ORI, Germany) which was then accustomed to 0.5 to acquire a constant cell population (10^8-10^9 CFU mL^{-1}) for inoculation (Afzal et al., 2020).

Crop management

Seed (ten) were sown in earthen pots (12 × 8 inches), and each pot was filled with 10 kg soil. The pots were watered to sustain 70% water-holding capacity. Throughout the experiment, the temperature, relative humidity and light intensity were 25±3/14±3°C (day/night), 35–75% (noon to midnight), and 400–1200 mmol photon m^{-2} s^{-1} respectively. After uniform emergence, five plants per pot were sustained by plugging out all surplus plants. The experimental soil, classified as sandy loam in texture, exhibited the following characteristics: 0.88% organic matter, pH of 8.02, electrical conductivity of 0.34 dS m^{-1}, 0.21 m mole 100 g^{-1} exchangeable sodium, 0.05% total N, 6.50 mg kg^{-1} P, 167 mg kg^{-1} Zn, 0.59 mg kg^{-1} B, and 6.77 mg kg^{-1} iron (Fe). To address the soil's nutrient profile, N (in the form of Ca $(NO_3)_2.4H_2O$), P (as KH_2PO_4), and potassium (as K_2SO_4) were applied at rates of 90, 60, and 25 mg kg^{-1} of soil, respectively, based on soil analysis results.

Soil analysis

Soil chemical properties were analyzed according to the International Center for Agricultural Research and Dry Areas [ICARDA], (2001). Total soil N contents were recorded using the Kjeldahl procedure after wet digestion. The sodium bicarbonate method (Olsen et al., 1954) amended by Olsen and Sommers (1982) was followed to measure available P. Extraction with ammonium acetate (Richards 1954) method was employed to record exchangeable K. To determine B, azomethine-H method (Bingham, 1982) was used.

Rhizosphere microbial count

The plants (tillering stage) were uprooted to collect the rhizosphere soil attached to the roots by tapping. After serial dilution, the soil sample suspensions were spread on tryptic soy agar plates, and count colony forming units (CFU) of (total) bacteria were observed following the dilution and plating method (Valentine et al., 2005). The plates were incubated at 28°C ± 2 for 48 hours. Colonies were counted and the colonization efficiency was obtained following the formula (Naveed et al., 2014):

$$\text{No. of colonies (CFU/ g dry weight)} = \frac{(\text{No. of colonies} \times \text{Dilution factor})}{\text{Volume of culture plate}}$$

Organic acids

The seedlings (50 DAS) along with the roots (5 seedlings per treatment) were moderately detached from the substrate and washed carefully with tap water. These seedlings were soaked for 6 hours in deionized water for exudate collection. After soaking the plants were taken out from the water, the roots and shoots were detached and weighed freshly. The obtained exudates were filtered (0.22 µm, Steriflip vacuum filter device, Millipore, ROTH, Karlsruhe, Germany) and their concentration was adjusted to 10 mL root exudate g^{-1} root fresh weight. These secretions were then stored in a refrigerator at -20°C until further processing. The profile of the root exudate was analyzed using an HPLC system (Agilent 1200, USA) equipped with an XDB-C 18 column (4.6 mm 6250 mm, Agilent, USA), which allowed the complete separation of nine standard organic acids present in plant roots secretions. The mobile phase used in HPLC contained 0.1% trifluoroacetic acid (A) and acetonitrile (B) with a 0 min gradient elution. The UV detector wavelength was fixed at 280 nm and a column temperature of 40 °C was sustained. Standard compounds only and in concoctions were separated and analyzed by chromatography and their retention times along with the main peaks of extracts were noted. Retention time and standard addition to the sample was used to classify the organic compounds in each fraction (Banwart et al., 1985).

Yield and related traits

Plants were harvested when ears turned golden, and grain became hard and golden. Yield related parameters and the grain yield were recorded following the standard procedures. At the last harvest, the number of fertile tillers from each pot was counted. After randomly selecting three ears from each pot and measuring the length with a measuring scale, the average ear length was calculated. In the same spike, spikelets per spike were counted and averaged. These spikes were then threshed by hand and the number of grains per spike was counted. From each treatment, 100 grains were parted and weighed up on a digital balance (USA, OHAUS, TS400S) and an average weight of 100-grains was calculated. Biological yields for each treatment were reported in grams/plot. Plants were manually threshed and the grain weight for each treatment was noted in grams on a digital scale (USA, OHAUS, TS400S).

Grain boron analysis

A dry ashing procedure was employed to prepare samples to record grain B concentration (Chapman et al., 1997). Samples for grains were completely dried at 70°C in an oven (IRMECO-GMbH Germany, Model IM-53). These desiccated samples were finely powdered in a crushing grinder (Cyclone Sample Mill, Model 3010-030). 1 g of individual grinded sample was retained in a crucible and then these crucibles were kept for 6 hours at 500°C in a muffle furnace (Thermo Scientific® FD1545M, range 100-1200 °C) for ashing. 10 mL of 0.36 N H_2SO_4 (Merck, purity: 98%) was incorporated in

every crucible and burnt samples were thoroughly mixed with H_2SO_4 and kept for 1 hour before filtration. After 1 hour these samples were filtered in a plastic vial via Whatman No. 1 filter paper, and then distilled water (IRMECO, Schwarzenbek Germany) was added to these samples to make a final volume of 50 mL. The filtered solution (2 mL) was mixed with a buffer solution (4 mL) [consisting of 25% ammonium acetate (25%), EDTA (1.5%) and acetic acid (12.5%), azomethine-H solution (4 mL) [consisting of azomethine-H (0.45%) and ascorbic acid (1%). Color development was allowed for 45 minutes and the amount of B was measured in each sample at 420 nm with a spectrophotometer (UV-4000, ORI, Germany) (Malekani and Cresser, 1998).

Statistical analysis

Fisher's analysis of variance procedure was employed to statistically analyze the collected data (Steel et al., 1997). Means of treatments were compared at the 5% probability level using the least significant difference (LSD). Graphical representation of data was performed on Microsoft Excel.

Results

Soil analysis

Boron application and *Bacillus* MN54 inoculation significantly affected the soil N contents at the tillering, booting, and anthesis stages (Fig.1). Seed of cultivar Faisalabad-2008 treated with *Bacillus* MN54 aided B seed priming and *Bacillus* MN54 aided hydropriming showed higher soil N contents at tillering and booting stages respectively. However, the seed of Lasani-2008 treated with *Bacillus* MN54 aided B seed priming gave the highest soil N contents at the anthesis stage (Fig. 1). Soil P contents at tillering and booting stages were also significantly affected by B and *Bacillus* MN54 inoculation methods (Fig. 2). The highest soil P contents were noted in the seed of Lasani-2008 treated with *Bacillus* MN54 aided B seed priming at the tillering stage likewise at the booting stage, the same treatment in Faisalabad-2008 resulted in higher soil P contents (Fig. 2). At all stages, B and *Bacillus* MN54 inoculation methods also significantly affected the soil K and B contents (Fig. 3,4). Seed of Faisalabad-2008 treated with *Bacillus* MN54 aided B seed priming resulted in higher soil K contents at all stages (Fig. 3). Seed priming with B with *Bacillus* MN54 resulted in higher soil B contents in Lasani-2008 at the tillering stage whereas in Faisalabad-2008 at the booting stage and the anthesis stages (Fig. 4).

Rhizosphere microbial count

The number of microbes per unit of rhizosphere soil was increased by all methods of *Bacillus* MN54 inoculation (Fig. 5). However, the highest count was recorded where seeds of both cultivars were treated with *Bacillus* MN54 aided B seed priming as well as where B and *Bacillus* MN54 were inoculated as soil application (Fig. 5).

Organic acids

Release of organic acid in root exudates of treated seed was increased by B and *Bacillus* MN54 application (Table 1). The highest pyruvic acid, tartaric acid, malic acid and citric acid production was documented where seeds were primed with B+ *Bacillus* MN54 in both cultivars (Table 1). Seed of Faisalabad-2008 primed with B+ *Bacillus* MN54 resulted in higher production of malonic acid (Table 1). Likewise, the production of oxaloacetic acid was also improved by seed priming with B+ *Bacillus* MN54 in both wheat cultivars (Table 1). Supply of B and *Bacillus* MN54 as seed priming in Lasani-2008 caused the production of succinic acid whereas all treatments could not affect the production of succinic acid (Table 1). Priming of Lasani-2008 seed with B and *Bacillus* MN54 also resulted in higher production of methylmalonic acid (Table 1).

Grain boron concentration

The application methods, including both B and *Bacillus* MN54 increased the grain B concentration in both wheat varieties (Fig. 6). Notably, the highest B concentration in wheat grain was observed in Lasani-2008 when plants received a foliar spray of B + *Bacillus* MN54. Similarly, substantial increases in grain B concentration were noted when Faisalabad-2008 was treated with B + *Bacillus* MN54, either as a soil application or foliar spray (Fig. 6).

Yield and yield related traits

During the first year of the study, a significant variation among treatments was observed for spike length, number of spikelets per spike and grain yield, while wheat cultivars varied significantly for fertile tillers, spike length, spikelets per spike, grains per spike, 100-grain weight and biological yield. Notably, Lasani-2008 exhibited the maximum number of productive tillers. The longest spike, the highest number of spikelets per spike, and number of grains per spike were recorded for Lasani-2008 when seeds were primed with B + *Bacillus* MN54 (Table 3). However, the highest 100-grain weight was noted in Faisalabad-2008 (Table 2). The highest grain yield was observed with B + *Bacillus* MN54 application as seed priming, seed coating and soil application (Table 2); while the highest biological yield was recorded in Lasani-2008 (Table 2).

In the second year of the study, treatments significantly differed in terms of various parameters. The cultivar and treatment interactions were found to significantly influence productive tillers, spike length, spikelets per spike, grains per spike, 100-grain weight and grain yield. The highest number of productive tillers were recorded in Lasani-2008 where seeds were coated with B. Maximum spike length was recorded in Lasani-2008 where seeds were treated with B and *Bacillus* MN54 as seed priming (Table 3). Seed priming with B+

Bacillus MN54 produced the highest number of spikelets per spike and grains per spike in Lasani-20008 (Table 2); while the highest 100-grain weight was recorded in Faisalabad-2008 where seeds were coated with B (Table 3). The highest grain yield was observed in seed priming with B and *Bacillus* MN54 (Table 2); while the same treatment produced the highest biological yield in Lasani-2008 (Table 2).

Discussion

The findings of this study have demonstrated improvements in soil nutrient content, microbial counts and organic acids, yield and grain B concentration by the combined application of *Bacillus* MN54 and B. In this study, the application of B and *Bacillus* MN54 through seed priming led to higher soil nutrient levels, particularly for N, P, K, and B, compared to other application methods. The availability of nutrients in the soil is known to be crucial for plant growth and productivity. While nutrients are present in the soil, the bioavailable forms of essential nutrients such as N, P, K, Zn, Fe and B are often limited in the rhizosphere due to various soil factors (Ngosong et al., 2022). It is well-documented that microorganisms enhance nutrient availability through processes like N fixation, solubilization of insoluble minerals and the production of organic acids and siderophores (Hamid et al., 2021; Chakraborti et al., 2022). Therefore, this enhancement can be attributed to the ability of *Bacillus* species to convert complex forms of these nutrients into simple, more accessible forms that plants can readily absorb and utilize (Bechtaoui et al., 2020). For instance, some *Bacillus sp*. releases ammonia from nitrogenous organics within the rhizosphere (Hayat et al., 2012). Additionally, some PGPRs such as diazotrophs are capable of fixing atmospheric N_2 due to the presence of the *nifH* gene and the production of nitrogenase (Ngosong et al., 2022). Furthermore, *Bacillus sp*. facilitates the conversion of inorganic phosphate into free phosphate by releasing phosphatases and organic acids (Behera et al., 2017), resulting in enhanced P levels in soil, as was observed in our study.

Likewise, the increase in soil K concentration can be ascribed to the role of some *Bacillus sp.* in the production of extracellular polysaccharides, which can break down K bearing minerals, releasing K into the soil solution (a well-documented process for K solubilization by PGPR) (Saxena et al., 2019). Moreover, our study has revealed that the availability of B in the soil-plant system can be improved through rhizospheric acidification induced by *Bacillus sp*. (Macias-Benitez et al., 2020). This acidification process is facilitated by the production of organic acids within the rhizosphere through PGPR (Masood, 2019) as well as through release of root exudates by plants (Sharma et al., 2020). Consequently, soil pH is temporarily reduced, leading to increased B availability (Masood, 2019; Khan et al., 2022). The observed increase in nutrient content with seed priming using *Bacillus* MN54 and B, compared to other application methods, may be due to the fact that seed priming activates physiological processes inside the seed, while preventing radicle and plumule emergence until sowing (Anitha et al., 2013). This early activation boosts the abundance of PGPR in the spermosphere (Taylor and Harman, 1990), additionally the microbial population within the seed remains protected from external environmental stresses (Mahmood et al., 2016). In contrast, microbial application through soil, foliar spraying, or seed coating requires higher volumes and can be detrimental to microbial viability due to exposure to sunlight and air-induced desiccation (Mahmood et al., 2016).

The application of *Bacillus* MN54 and B through seed priming also led to an increase in rhizosphere microbial populations compared to other application methods. Seed priming directly introduces beneficial microbes onto or into the seed, enabling early colonization of the rhizosphere as the seed begins to germinate (Mahmood et al., 2016). This synchronized microbial establishment with root emergence provides microbes with a competitive advantage in colonizing the root zone. During priming, the moist conditions activate the microbes, allowing them to multiply or become more physiologically active before sowing. This early activation enhances the survival and competitiveness of the microbes once in the soil (Chakraborti et al., 2022). Compared to soil or foliar applications, seed priming offers a more protective environment for microbes, shielding them from desiccation, UV radiation, and antagonistic soil microorganisms (Gogoi et al., 2018).

The seed coat and the developing root zone create an ideal niche for microbial survival and growth. As the seed germinates, root exudates rich in sugars, amino acids, and organic acids are released, acting as chemical cues that attract and nourish microbial communities (Bashan et al., 2004). Microbes introduced via seed priming are well-positioned to utilize these exudates, promoting their growth and activity in the rhizosphere. This study also observed elevated levels of organic acids in the rhizosphere, particularly in the treatment where *Bacillus* MN54 and B were applied via seed priming. Previous research has shown that PGPR enhance organic acid concentrations in the rhizosphere (García-López and Delgado, 2016; Pii et al., 2015; Aras et al., 2018). As discussed earlier, the combined application of B and *Bacillus* MN54 through seed priming significantly boosted microbial populations in the rhizosphere.

This increase in microbial activity likely contributed to the higher organic acid content in the soil, as microbial metabolism is closely associated with organic acid production. A denser microbial community also promotes early root colonization and efficient use of root exudates, fostering a rhizospheric environment conducive to greater organic acid synthesis. Additionally, the results revealed that this treatment led to an increased accumulation of B in the grain. This could be attributed, firstly, to the ability of PGPR to promote root development and improve nutrient uptake efficiency (Turan et al., 2021), and secondly, to the role of seed priming in stimulating early metabolic and hormonal processes that enhance root growth and functionality (Rehman et al., 2021).

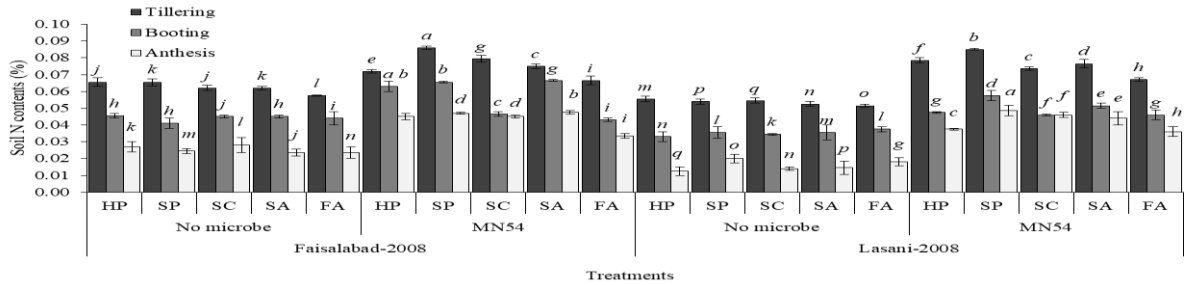

Fig. 1 Effect of different boron application methods, with or without *Bacillus* MN54 addition on soil nitrogen contents.
HP = Hydropriming; SP = seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

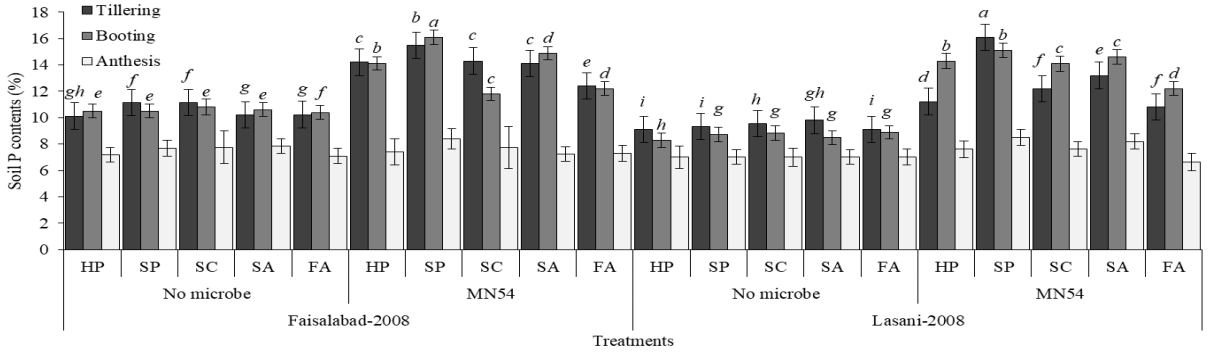

Fig. 2 Effect of different boron application methods, with or without *Bacillus* MN54 addition on soil phosphorous contents. HP = Hydropriming; SP = seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

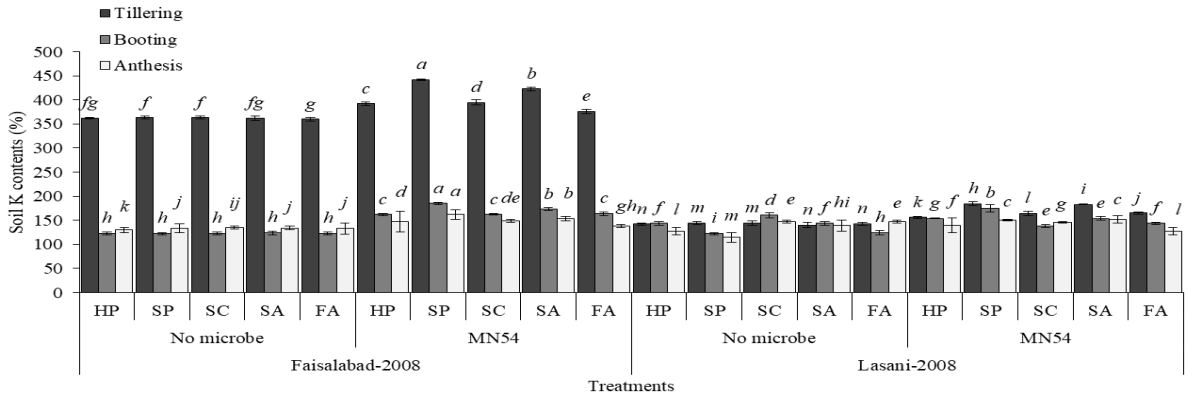

Fig. 3 Effect of different boron application methods, with or without *Bacillus* MN54 addition on soil potassium contents. HP = Hydropriming; SP = seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

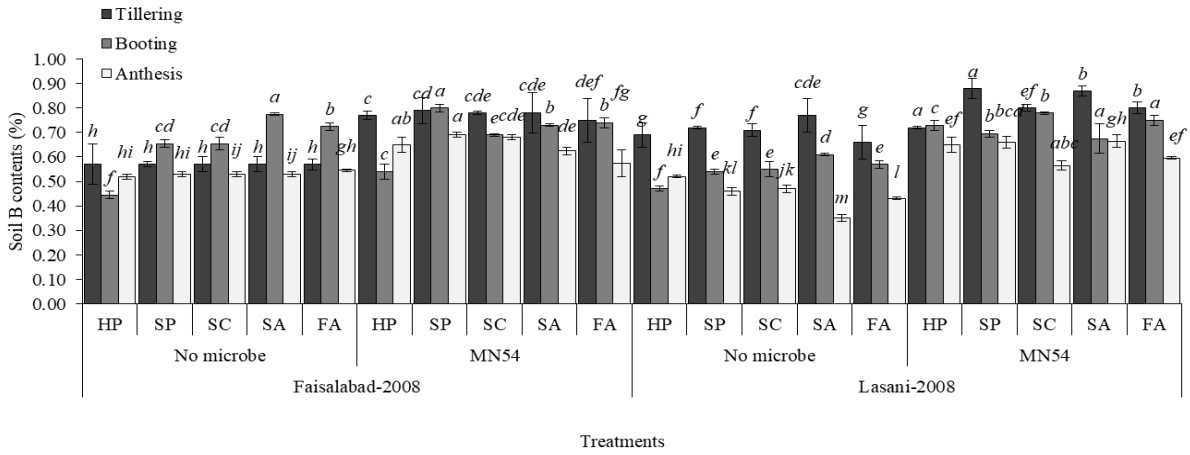

Fig. 4 Effect of different boron application methods, with or without *Bacillus* MN54 addition on soil boron contents. HP = Hydropriming; SP = seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

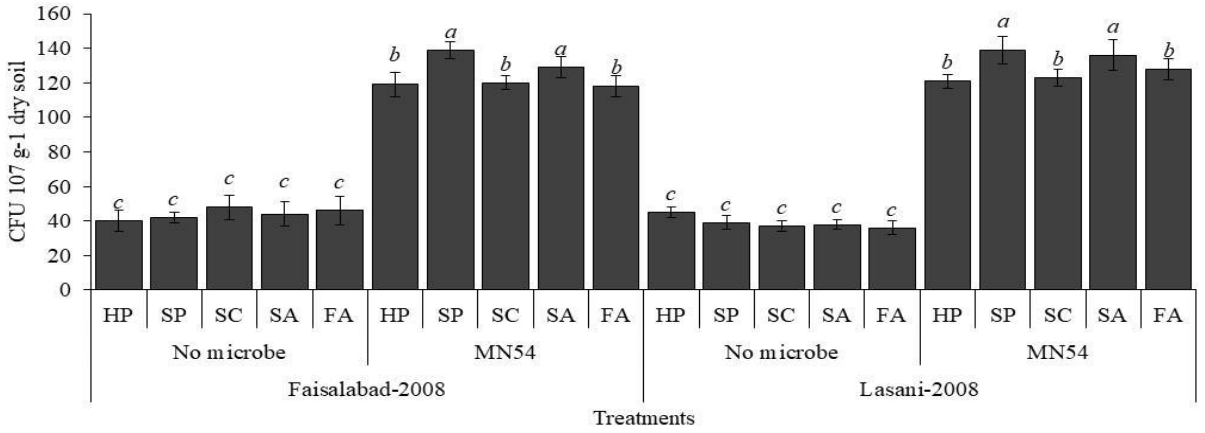

Fig. 5 Effect of different boron application methods, with or without *Bacillus* MN54 addition on rhizosphere microbial count of wheat cultivars Faisalabad-2008 and Lasani-2008. HP = Hydropriming; SP = Seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

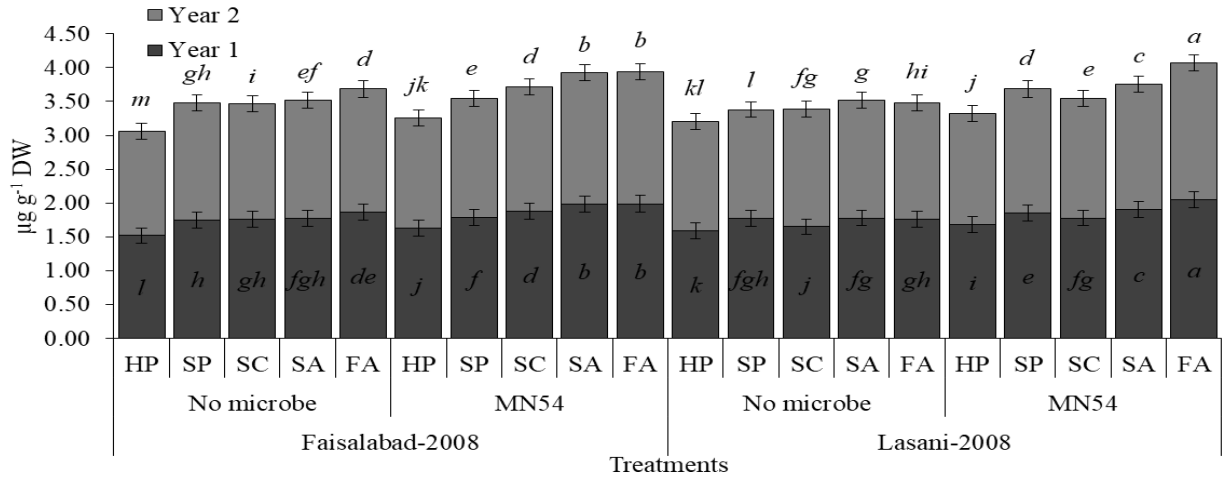

Fig. 6 Effect of different B application methods, with or without *Bacillus* MN54 addition, on grain boron concentration of wheat cultivars Faisalabad-2008 and Lasani-08. HP = Hydropriming; SP = Seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application

Consequently, the improved root system, combined with Bacillus MN54 application, likely enhanced soil B uptake, resulting in greater B accumulation in grains. The application of *Bacillus* MN54 led to improvements in grain yield and related parameters of wheat. This enhancement can be attributed to the ability of *Bacillus* MN54 to produce plant growth substances and dissolve insoluble minerals, along with former plant growth and nutrient augmentation abilities shown in the results argued before. Application of B further amplified the positive impact on yield related traits of wheat, such as leaf number and tiller number, which are closely interrelated. It is well-documented that B application has the potential to enhance these traits (Rehman et al., 2015).

The significant role of B in various cellular processes cannot be overlooked. Notably, B is vital for maintaining cell membrane function and integrity (Brown et al., 2002). Additionally, B is involved in cell wall formation (Riaz et al., 2021), contributes to the breakdown of essential biological compounds (Goldbach et al., 2001), and plays a role in cell wall synthesis and cell division (Mouhtaridou et al., 2004). These diverse cellular functions of B may account for the observed improvements in tillering when wheat seeds are primed with B. The application of B in our study led to increased spike length, which can be attributed to the involvement of B in cell elongation and division (Liao and Weijers, 2018). The absence of B has profound implications for plant cell wall formation, as it disrupts cell's ability to regulate mitosis, affecting cell division (Sakamoto et al., 2021). Boron plays a pivotal role in regulating the binding of two RG-II polysaccharides in the plant cell wall (O'Neill et al., 2004). This RG-II-B complex influences plant cell wall relaxation/elongation by regulating porosity (Miwa et al., 2013) and tensile strength (Ryden et al., 2003). Furthermore, B deficiency impairs the functioning of genes responsible for regulating cell wall amending enzymes (Tenhaken, 2015) and consequently restricts cell elongation (Camacho-Cristobal et al., 2008).

Seed priming with B in combination with *Bacillus* MN54 led to an increase in the number of spikelets per spike and the number of grains in each spike, primarily due to longer spikes. Moreover, the presence or absence of B significantly impacts seed setting in wheat. Boron deficiency causes irregularities in stigma function, pollen growth (Mokwala, 2018), and pollen grain viability (Penaloza & Toloza, 2018), leading to a reduction in fertilization success. This deficiency also disrupted the assembly of the pollen tube cell wall, resulting in tube rupture and suppressed elongation (Gonzalez-Fontes, 2020).

This study also reported a higher 100-grain weight when B + *Bacillus* MN54 were applied as seed priming. This improvement may be ascribed to the vigorous growth and early establishment of primed seeds, coupled with role of B in photosynthesis (Chen et al., 2022). B is crucial for sugar breakdown and facilitates the movement of sugars from source to sink tissues, contributing to an increase in grain weight following B + *Bacillus* MN54 application (Reddy et al., 2003). However, B deficiency can lead to anomalous accumulation of B in the leaves, disrupting these vital processes, and reducing photosynthesis efficiency (Wimmer & Eichert, 2013). Therefore, the improved grain weight observed in our study can be attributed to the combined influence of B on growth dynamics and sugar transport, bolstered by the positive effects of MN54 application. The application of B + *Bacillus* MN54 resulted in a higher grain yield in this study, which can be attributed to its positive influence on various wheat yield-related factors, including the number of tillers, number of grains per ear and 100-grain weight, all of which showed improvement due to B + *Bacillus* MN54 seed priming. Boron deficiency is known to impede leaf expansion, leading to reduced stomatal density and smaller stomatal apertures (Furlani et al., 2003). It disrupts the structure and functioning of chloroplasts, particularly the thylakoid membrane, leading to limited CO_2 assimilation and, consequently, reduced plant photosynthesis (Pandey & Pandey, 2008). In our study, the enhancements in wheat yield-related traits with B and *Bacillus* MN54 application suggest an increase in photosynthetic activity, likely due to the improved B availability. Furthermore, the higher grain yield could also be attributed to increased photo-assimilate accumulation facilitated by B (Arif et al., 2006).

Conclusion

Application of B and *Bacillus* MN54 using various methods (priming, soil coating, and foliar application) led to significant improvements in soil nutrient content, microbial populations, grain yield, grain B concentration, and the production of organic acids in wheat root exudates. Notably, the combination of B and *Bacillus* MN54 as seed priming and soil application resulted in the highest yields, increased soil nutrient content, and elevated organic acid levels in wheat root exudates. On the other hand, the application of B and *Bacillus* MN54 as foliar and soil treatments proved to be most effective in enhancing grain B concentrations in bread wheat.

Table 1 Effect of different B application methods, with or without *Bacillus* MN54 addition, on release of organic acids (mg ml^{-1}) in wheat cultivars Faisalabad-2008 and Lasani-2008

Treatments		FSD-08	LS-08	FSD-08	LS-08	FSD-08	LS-08	FSD-08	LS-08
		Pyruvic acid		Tartaric acid		Malice acid		Citric acid	
No *Bacillus* MN54	HP	1.38 ±0.14	36.3 ±1.63	92.5 ±9.25	74.3 ±8.46	6.32 ±0.63	n.d	n.d	n.d
	SP	1.06 ±0.11	40.9 ±4.09	92.5 ±9.25	75.2 ±17.5	9.75 ±1.98	6.32 ±0.63	34.2 ± 3.42	42.4±4.24
	SC	1.19 ±0.32	41.6 ±4.16	92.8 ±29.8	78.9 ±7.89	11.77 ± 1.18	n.d	55.6 ± 5.56	n.d
	SA	1.53 ± 0.45	42.2 ± 3.22	94.6 ±83.5	76.8 ±92.7	6.00 ±0.60	n.d	n.d	19.4 ± 1.94
	FA	0.24 ±0.02	35.2 ±3.52	91.7 ±34.1	73.1 ± 19.3	n.d	n.d	n.d	54.1± 5.41
Bacillus MN54	HP	80.8 ±6.08	41.6 ±4.16	1148 ±115	484 ±3.92	40.3 ± 4.03	20.5 ±0.40	64.9 ±7.06	271 ± 7.88
	SP	92.8 ±8.32	163.2 ± 4.48	2472 ± 247	2330 ±14	44.6 ± 4.46	23.7 ±0.20	789 ± 78.9	324 ± 6.00
	SC	89.6 ±8.96	55.7 ±5.57	1398 ±139	806 ±11.5	18.9 ±1.90	4.74 ± .19	210 ± 3.42	194 ± 2.11
	SA	83.2 ±9.28	44.8 ±16.3	301 ±30.1	283 ± 24.7	19.8 ±1.98	3.56 ±0.45	103 ± 10.3	138 ± 0.34
	FA	20.5 ±2.05	32.5 ±3.25	392 ±39.2	101 ±3.01	14.2 ±1.42	n.d	n.d	23.5 ± 1.03
		Melonic acid		Oxaloacetic acid		Succinic acid		Methylmelonic acid	
No *Bacillus* MN54	HP	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	SP	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.05±0.02
	SC	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	SA	n.d	n.d	n.d	2.86±0.13	n.d	n.d	n.d	n.d
	FA	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Bacillus MN54	HP	n.d	n.d	6.46±0.24	7.04±0.19	n.d	n.d	n.d	n.d
	SP	2.45±0.25	n.d	7.21±0.31	5.89±0.13	n.d	5.49±0.24	0.07±0.02	0.22±0.01
	SC	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.06±0.01
	SA	1.12±0.11	n.d	n.d	n.d	n.d	n.d	n.d	0.13±0.01
	FA	0.06±0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.05±0.01

Values are mean± standard error of means; n.d., Not detected; HP= Hydropriming; SP= Seed priming; SC= Seed coating; SA= Soil application; FA= Foliar application

Table 2 Effect of different B application methods, with or without *Bacillus* MN54 addition, on yield and related traits of wheat cultivars Faisalabad-2008 and Lasani-2008

Treatments	Productive tillers (m^{-2})		Spike length (cm)		Spikelets per spike		Grains per spike		100-grains weight (g)		Grain yield (g pot^{-1})		Biological yield (g pot^{-1})	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
							Cultivar (C)							
Faisalabad-2008	2.26 B	2.88 B	6.21 B	9.29 B	9.48 B	16.25 B	33.49 B	46.44 B	3.10 A	45.55 A	3.04	4.58	9.14 B	8.98 B
Lasani-2008	2.64 A	3.02 A	6.58 A	9.45 A	10.53 A	16.53 A	71.31 A	47.23 A	2.76 B	43.91 B	3.26	4.70	12.36 A	12.23 A
LSD P≤0.05	0.16	0.37	0.24	0.11	0.48	0.19	2.03	0.54	0.14	0.77	NS	NS	1.45	0.76
Treatments (T)														
No HP	2.37	2.70	6.04	9.07	8.83	15.87	30.33	45.33	2.83	44.84	2.49	4.54	8.80	8.91

Bacillus			DE	C	C	C	C	D	C			B	C		D
MN54	SP	2.54	3.32 B	6.11 BC	9.37 B	9.96 AB	16.40 B	34.67 CD	46.41 BC	3.01	44.43	3.01 AB	4.84 C	10.87	10.16 BCD
	SC	2.29	2.55 E	6.13 BC	9.44 B	10.25 AB	16.29 BC	38.83 A-D	46.85 B	3.00	45.39	3.79 A	4.83 C	11.16	11.09 AB
	SA	2.50	2.82 D	6.04 C	9.11 C	9.54 BC	16.24 BC	29.21 D	46.54 BC	2.91	43.88	2.55 B	3.92 D	8.82	9.21 CD
	FA	2.59	3.10 C	6.33 BC	9.28 BC	9.60 BC	15.95 C	29.46 D	45.57 C	2.76	43.80	2.64 B	4.17 D	9.73	8.74 D
Bacillus MN54	HP	2.39	2.59 E	6.59 AB	9.46 AB	10.29 AB	16.56 AB	35.67 BCD	47.31 AB	2.92	44.46	3.24 AB	4.12 D	9.73	11.83 AB
	SP	2.61	3.78 A	7.02 A	9.70 A	10.71 A	16.97 A	46.21 A	48.48 A	3.09	45.72	4.10 A	6.09 A	12.77	12.30 A
	SC	2.33	2.55 E	6.49 ABC	9.45 AB	10.58 AB	16.55 AB	45.46 AB	47.27 AB	2.92	45.26	3.88 A	4.63 C	12.22	11.10 AB
	SA	2.61	3.11 C	6.65 AB	9.48 AB	10.38 AB	16.59 AB	41.04 ABC	47.41 AB	2.97	45.42	3.85 A	5.21 B	12.47	11.91 AB
	FA	2.28	3.00 C	6.54 ABC	9.31 BC	9.92 AB	16.52 B	44.33 ABC	47.20 B	2.90	44.06	3.34 AB	4.06 D	10.80	10.81 ABC
LSD P≤0.05		NS	0.17	0.54	0.24	1.07	0.42	2.03	1.22	NS	NS	1.11	0.32	N S	1.69

HP = Hydropriming; SP = Seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application; C = cultivar; M = Microbe; T = treatment; NS = non-significant

Table 3 Effect of interaction of different B application methods, with or without *Bacillus* MN54 addition, on yield and related traits of wheat cultivars Faisalabad-2008 and Lasani-2008

Treatments			Productive tillers (m^{-2})		Spike length (cm)		Spikelets per spike		Grains per spike		100-grains weight (g)		Grain yield (g pot^{-1})		Biological yield (g pot^{-1})	
			Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
Faisalabad-2008	No *Bacillus* MN54	HP	2.61	2.59 e-h	5.76	9.24 e-j	8.08 g	16.17 d-j	26.33	46.19 e-j	3.22	44.50 d-h	2.48	3.74 i	7.14	7.21
		SP	2.33	2.69 d-g	6	9.08 hij	9.33 d-g	15.89 hij	30.42	46.86 c-h	3.05	42.45 hi	2.88	5.64 bcd	8.68	7.94
		SC	2.28	2.38 h	5.63	9.36 c-h	10.17 a-e	16.19 e-i	36.65	45.40 hij	3.06	48.68 a	2.92	5.50 cd	9.82	9.86
		SA	2.68	2.61 d-h	5.78	9.31 d-h	8.42 fg	16.40 c-h	21.33	46.26 e-i	2.91	42.03 i	2.09	3.82 hi	6.83	8.89
		FA	2.33	3.40 b	6.03	9.37 c-h	8.11 g	16.29d-h	28.33	46.54 d-h	2.98	46.09 b-e	2.74	4.23 fgh	7.67	6.49
	Bacillus MN54	HP	2.41	2.39 h	6.08	9.29 e-h	9.08 efg	16.26 e-h	33.25	46.45 e-h	3.14	42.93 ghi	3.02	3.76 i	9.49	9.93
		SP	2.34	3.48 b	7.04	9.43 c-g	11.00 abc	16.50 c-g	47.25	47.14 c-g	3.13	43.81 e-i	3.48	5.81 bc	11.82	11.64

		SC	2.12	2.56 fgh	6.54	9.13 g-j	10.58 a-e	15.98 g-j	37.83	45.65 g-j	3.16	43.02 ghi	3.47	4.78 e	10.31	8.58
		SA	2.23	3.37b	6.67	9.43 c-g	9.67 b-f	16.50c-g	31.5	47.14 c-g	3.15	43.60 f-i	3.87	4.37 efg	9.81	9.33
		FA	2.03	3.36 b	6.60	9.25 e-i	10.33 a-e	16.38 c-h	44.42	46.81 c-h	3.21	41.98 i	3.44	4.14 f-i	9.78	9.95
Lasani-2008	No *Bacillus* MN54	HP	2.48	0.24	6.33	8.89 j	9.58 b-g	15.56 j	34.33	44.47 j	2.45	45.17 c-g	2.51	5.34 d	10.46	10.62
		SP	2.97	2.82 cde	6.23	9.66 abc	10.58 a-e	16.91 abc	38.92	45.95 f-j	2.96	46.42 a-d	3.14	4.03 f-i	13.06	12.36
		SC	2.47	3.94 a	6.64	9.52 b-f	10.33 a-e	16.39 c-h	41.00	48.30 abc	2.93	42.10 hi	4.2	4.17 f-i	12.51	12.31
		SA	2.32	2.72 d-g	6.3	8.92 ij	10.67 a-d	16.08 f-j	37.08	46.82 c-h	2.91	45.73 b-f	3.01	4.01 ghi	11.00	9.53
		FA	2.85	3.03 c	6.63	9.19 f-j	11.08 ab	15.61 ij	30.58	44.60 ij	2.54	41.51 i	2.54	4.10 f-i	11.78	10.99
	Bacillus MN54	HP	2.37	2.80 c-f	7.11	9.64 a-d	11.50 a	16.86 a-d	38.08	48.18 a-d	2.7	46.00 b-f	2.47	4.47 ef	9.96	13.73
		SP	2.87	2.78 d-g	7.01	9.97 a	10.42 a-e	17.44 a	45.17	49.84 a	3.05	47.63 ab	4.19	6.36 a	13.71	12.97
		SC	2.54	4.08 a	6.44	9.78 ab	10.58 a-e	17.11 ab	53.08	48.90 ab	2.68	47.52 abc	3.44	4.47 ef	14.12	13.62
		SA	2.99	2.54 gh	6.63	9.54 b-e	11.08 ab	16.69 b-e	50.58	47.68 b-e	2.79	47.23 abc	3.84	6.05 ab	15.12	14.48
		FA	2.53	2.85 cd	6.48	9.36 c-h	9.50 c-g	16.66 b-f	44.25	47.59 b-f	2.59	46.15 b-e	3.23	3.99 ghi	11.93	11.66
LSD P≤0.05			NS	2.64	NS	0.34	1.51	0.60	NS	1.72	NS	2.43	NS	0.45	NS	NS

HP = Hydropriming; SP = Seed priming; SC = Seed coating; SA = Soil application; FA = Foliar application; C = Cultivar; M = Microbe; T = Treatment; NS = Non-significant

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