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Alleviation of Salt Stress in Wheat Seedlings via Multifunctional *Bacillus aryabhattai* PM34: An *In-Vitro* Study

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Abstract: Plant growth-promoting rhizobacteria play a substantial role in plant growth and development under biotic and abiotic stress conditions. However, understanding about the functional role of rhizobacterial strains for wheat growth under salt stress remains largely unknown. Here we investigated the antagonistic bacterial strain *Bacillus aryabhattai* PM34 inhabiting ACC deaminase and exopolysaccharide producing ability to ameliorate salinity stress in wheat seedlings under in vitro conditions. The strain PM34 was isolated from the potato rhizosphere and screened for different PGP traits comprising nitrogen fixation, potassium, zinc solubilization, indole acetic acid, siderophore, and ammonia production, along with various extracellular enzyme activities. The strain PM34 showed significant tolerance towards both abiotic stresses including salt stress (NaCl 2 M), heavy metal (nickel, 100 ppm, and cadmium, 300 ppm), heat stress (60 °C), and biotic stress through mycelial inhibition of *Rhizoctonia solani* (43%) and *Fusarium solani* (41%). The PCR detection of *ituC*, *nifH*, and *acds* genes coding for iturin, nitrogenase, and ACC deaminase enzyme indicated the potential of strain PM34 for plant growth promotion and stress tolerance. In the in vitro experiment, NaCl (2 M) decreased the wheat growth while the inoculation of strain PM34 enhanced the germination% (48%), root length (76%), shoot length (75%), fresh biomass (79%), and dry biomass (87%) over to un-inoculated control under 2M NaCl level. The results of experiments depicted the ability of antagonistic bacterial strain *Bacillus aryabhattai* PM34 to augment salt stress tolerance when inoculated to wheat plants under saline environment.

Keywords: salinity stress; biocontrol; PGPR; *Bacillus aryabhattai*

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1. Introduction

Wheat (*Triticum aestivum*) is one of the widely grown food crops globally with an annual production of 760 million tons in 2019–2020 [1]. It fulfills the nutritional requirements of 21% of the world's population [2]. However, the world population is increasing day by day, and is anticipated to reach up to 9.6 billion, with food demand doubled by 2050 (https://population.un.org/wpp/2019). Moreover, wheat crop encounters various abiotic (e.g., temperature, drought, heavy metals, and salt stress) and biotic (e.g., insect attack, weed, and disease infestation) stresses during the developmental stages. These environmental stresses deteriorate the plant's major metabolic activities, such as decreased

photosynthesis, cell wall injury, the buildup of compatible solutes including soluble sugars, proline, and electrolyte leakage, all of which lead to retarded germination and growth [3]. Among all abiotic stresses, salinity stress has potential impact on limiting plant growth, photosynthesis activity, and protein synthesis, and negatively affects lipid metabolism which devastates plant growth and reduces yield production [4,5]. It has been reported that 1125 million hectares land is salt-affected worldwide, reducing average yields by 50–80% [6,7]. Besides, among biotic stresses, the phytopathogens are responsible for yield losses about 20–40% and loss of USD 40 billion worldwide [8]. Fungal pathogens including *Rhizoctonia solani* (*R. solani*) and *Fusarium solani* (*F. solani*) are the most damaging soil-born plant pathogens which cause diseases in various crops throughout the world and significantly reduce the crop yield [9,10]. Therefore, environmentally friendly strategies are critically required to minimize the massive impact of salinity on crop production.

So far, various practices are recommended to counteract the problem of soil salinity, such as saline soils being reclaimed through gypsum ($CaSO_4.2H_2O$) application This practice has some environmental and economic risks owing to its chemical nature and increasing production costs for farmers. Therefore, it is a matter of great interest to use low-cost and environmentally friendly organic materials [4,11–13] for reclamation of saline soils. For example, the root-associated microbes have a close relationship with one another and can play a significant role in key physiological processes, including nutrient acquisition and plant stress (biotic and abiotic) tolerance [4]. Plant growth-promoting rhizobacteria (PGPR) have been used extensively to increase plant growth and induce resistance in plants to biotic and abiotic stresses. These PGPR have significant capacity to improve sustainable crop production under abiotic and biotic stress conditions [14,15] through the stress tolerance mechanism. By altering plant physiology, the PGPR induces systemic immunity to abiotic stress factors such as salinity, drought, and heavy metals [16]. Furthermore, a few PGPR strains colonizing plant roots cause an induced systemic resistance (ISR) reaction that works systemically across the plant body and is often effective against a wide range of plant pathogens. These beneficial rhizobacteria may be an excellent choice for improving crop plant salinity and disease resistance.

Various studies have found that PGPRs have substantial impact in improving crop growth owing to salt stress resistance [4,17]. Numerous bacterial genera have been tested for their capacity to develop resistance against various stresses and to solubilize nutrients as well as bio-control potential in crop production systems [18]. However, application of the rhizobacteria from lab to greenhouse or field conditions face the prevalence of various stress factors including salinity, high temperature, and drought where the survival of the applied rhizobacteria becomes a major apprehension [19]. Thus, in such prevailing situation, these challenges can be controlled through application of capable multi-stress tolerant PGPR.

The screening of multi-stress resistant PGPR is critical for their practical use under field conditions to achieve sustainable crop production. Thus, the objectives of our present research were as follows: (1) To isolate the bacteria equipped with plant growth-promoting traits, salt stress tolerance, and bio-control capacity; (2) to identify the bacterial isolate PM34 using 16S rRNA sequencing, and to test the bacterial isolate PM34 to ameliorate abiotic (salt) stress conditions and biotic (*R. solani* and *F. solani*) stress.

2. Materials and Methods

2.1. Bacterial Strain Isolation and Fungal Strain Acquisition

The rhizosphere soil samples of potato were collected from potato cultivated in Chitral, Pakistan (35.991119° N, 71.557720° E) and the strain PM34 was isolated from the rhizosphere soil through serial dilution method [20]. Both fungus *Rhizoctonia solani* and *Fusarium solani* were obtained from Crop Disease Research Institute, NARC, Islamabad, Pakistan (33.677113° N, 73.139111° E). Pathogenicity test for the fungal strains was done by following [21].

2.2. Plant Growth-Promoting Traits of Strain PM34

Bacterial strain PM34 with 109 CFU/mL was used throughout the study. Atmospheric nitrogen fixation capability of the strain PM34 was assessed by inoculating in nitrogen-free medium [9]. Alexandrov agar medium (g L^{-1} glucose, 5.0; $MgSO_4$, 0.5, $CaCO_3$, 0.1; FeCl2, 0.006; $Ca_3(PO_4)_2$, 2.0; insoluble potassium source (Feldspar), 3.0, and agar 15 with pH 7.5) was used to check the potassium solubilization activity of the strain PM34. For this purpose, the single bacterial colony of the isolate PM34 was inoculated on the Alexandrov agar medium plates in triplicate and the plates were incubated at 32 °C for 7 days (d). The halo zone surrounding the bacterial colony indicated solubilization of potassium [22]. For zinc solubilization, the respective zinc medium (g L^{-1}, $(NH_4)_2SO_4$, 1.0; glucose, 10; KCl, 0.2; $MgSO_4$, 0.2; K_2HPO_4, 0.1 and agar 15 at pH 7.0, supplemented with insoluble zinc oxide or zinc carbonate), was prepared and the bacterial colony was inoculated on the plates containing the insoluble Zn medium. Spot inoculated plates were incubated at 32 °C for 7 d [23]. LB-broth medium (Luria-Bertani) amended with L-tryptophan (1.0 g L^{-1}) was used for indole-3-acetic acid (IAA) assay. After inoculation, the samples were kept on shaking at 28 °C and 120 rpm for 3 to 4 d. The appearance of cherry-red colored ring upon addition of 5 drops Kovacs reagents was the indication of IAA production [24]. Siderophore production by bacterium was assessed using Chrome azurol S blue agar plates, which has firmed complexes of HDTMA with ferric iron that generates dark blue color. Upon bacterial inoculation, iron chelator like siderophore produces by bacteria removes iron from the dye, and the color shifts from blue to yellow-orange. The respective medium was prepared by suspending 60.5 mg Chrome azurol S in 50 mL of deionized water and was added to 10 mL iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). The prepared solution was mixed with another solution prepared by dissolving hexadecyl trimethyl ammonium in 40 mL of deionized water. After inoculation, plates were incubated at 32 °C for 2 to 3 d. Formation of yellow-orange-colored zone surrounding the colony was an indication of siderophore production [16]. To check ammonia production, the strain PM34 was inoculated in 10 mL of peptone water medium prepared by dissolving 15 g peptone water in 1 L and incubated on shaking at 28 °C for 2 to 3 d. The appearance of dark brown color upon addition of 0.5 mL of Nessler's reagent ($HgCl_2$, 10 g; KI, 7 g; NaOH, 16 g; water, 100 mL with pH 13.2 ± 0.05) to test tube was indication of ammonia production [25].

2.3. Extracellular Enzymes, ACC Deaminase, and Exopolysaccharide (EPS) Production by Strain PM34

ACC-deaminase enzyme detection was assessed by utilizing ACC as the only supply of nitrogen using the procedure adopted previously [26]. The ability of the strain PM34 to produce pectinase, protease, amylase, and catalase was evaluated followed by [16]. Cellulase and chitinase activity of the strain PM34 was assessed by following [27]. Chitinase enzyme production assay was performed using a basal production media (g L^{-1} colloidal chitin, 3; yeast extract, 3; diammonium sulphate, 1.0; potassium dihydrogen phosphate, 1.36; hydrated magnesium sulphate, 0.5 at 7.2). For this purpose, single bacterial colony was inoculated on the plates containing the basal production medium in triplicate and the inoculated plates were incubated for 7d at 30 °C. The emergence of clear-cut halo zones around the bacterial colonies showed the production of chitinase by the bacterial isolate. To observe the production of EPS by the isolate PM34, the bacterial isolate was inoculated on the plates containing ATCC medium No.14 and the plates were incubated at 32 °C for 3 d. Slimy growth of bacteria on the ATCC plates was an indication of EPS production by the bacterial strain PM34 [4].

2.4. Molecular Profiling (Amplification of 16S rRNA, nifH, ituC, and Acds Gene

The genomic DNA extraction of strain PM34 was executed [28]. Primers used to amplify the 16S rRNA, *nifH*, *ituC*, and *acds* gene are summarized in Table 1. The reaction mixture consisted of 50 µL volume containing 2 µL genomic DNA, 5 µL 10 × PCR buffer, 200 µM dNTPs, 100 µM of each primer, and 1.25 U Top Taq DNA polymerase. For 16S

rRNA, PCR products were supplied to Macrogen, Korea for sequencing. Homology of the sequence was determined by BLAST analysis on the NCBI database. Similar sequences were downloaded, and phylogenetic tree was constructed using MEGA 6.0 software. For amplification of iturin gene (*ituC*) from strain PM34, the conditions retained included 95 °C for 4 min next 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 70 °C for 1 min with the last stage (extension) for 5 min at 70 °C. For *nifH* gene amplification, PCR reaction conditions included initial denaturation at 97 °C for 3 min; 97 °C, 55 °C for 50 s, and 72 °C for 35 s, 40 cycles; final extension 72 °C for 5 min. For ACC deaminase enzyme (*acds*) PCR reaction conditions included initial denaturation at 94 °C for 180 sec, subsequent denaturation for 60 s at 94 °C (30 cycles), annealing for 60 s at 58 °C, extension for 180 s at 72 °C, and a final primer extension at 72 °C for 30 s. Two percent agarose gel was used to analyze for PCR products using gel electrophoresis.

Table 1. Primers used for the PCR detection of 16S rRNA, *nifH*, *ituC*, and *acds* gene in strain PM34.

Genes	Primers	Primer Sequence (5′-3′)	PCR Product Size Expected/Detected (pb)	References
16S rRNA	27F 1492R	AGAGTTTGATC AC TGGCTCAG CGG CTTACCTTGTTACGACTT	1500/yes	[29]
ituC	ITUC-F1 ITUC-R1	CCCCCTCGG TCAAGTGAATA TTGGTTAAGCCCTGATGCTC	594/yes	[18]
nifH	nif h-F1 nif h-R1	TATGATCCAAAAGCAGA ATAGCCATCATTTCACC	360/yes	[30]
acds	acds-F1 acds-R1	GGCAAGGTCGACATCTATGC GGCTTGCCATTCAGCTATG	560/yes	[31]

2.5. Abiotic and Biotic Stress Tolerance Assay

The bacterial isolate PM34 was tested for its tolerance level against the different levels of NaCl. The isolate was inoculated in nutrient broth with two NaCl levels of 0, 1, and 2 M. The samples were incubated for 7 days at 32 °C in a shaking incubator. The optical density (OD_{600}) of samples was calculated and a growth curve was developed to predict bacterial growth daily for 7 days [16]. The nutrient agar medium was supplemented with different heavy metal concentrations for the heavy metal tolerance (Nickel and Cadmium 100–500 mg/L). Then strain PM34 was further evaluated in nutrient broth with 100 mg/L Ni and 300 mg/L Cd and OD_{600} was noted over 6 days. The strain PM34 was incubated at various temperature ranges, i.e., 40–60 °C for 6 d. More than 0.2 OD_{600} of bacterial culture was found to be thermo-tolerant at 45 °C [29–32]. To check the antagonistic potential of strain PM34 dual culture technique was applied. A fungal disc of 5 mm was positioned on dual culture medium (PDA and LB = 1:1) and bacterial strain was streaked at an equal distance beside the fungus plug. For control, the plates were inoculated with only fungal plug. The inoculated plates were kept at 28 ± 2 °C for 7 days. Inhibition of fungal mycelium was evaluated through following equation:

$$I = 1 - T/C \times 100 \quad (1)$$

The radial expansion of the fungus in control is denoted by "C," and the radial expansion of the fungal pathogen next to the PM34 strain is denoted by "T."

2.6. In Vitro Experiment

Seeds of wheat (Variety Pakistan-13) were obtained from NARC, Islamabad, Pakistan (33.677113° N, 73.139111° E) and seed sterilization was carried out through soaking in 75% ethanol and 0.1% $HgCl_2$ for 5 and 1 min, respectively. Surface sterilization of soaked seeds was done by washing thrice in distilled water [4]. The isolate PM34 was grown in LB broth medium for 3 d and harvested at log phase, centrifuged (10,000 rpm), washed with 0.80% (*w*/*v*) NaCl, and resuspended in double distilled water, and the strength of the inoculum (10^9 CFU/mL; OD_{600} = 1.0) were maintained. Then 2 mL of the bacterial culture was applied to wheat seeds for 2–4 h in respective treatments. Wheat seeds (10 seeds/plate) treated with

B. aryabhattai PM34 were put in sterilized Petri plates lined with double layer of Whatman No.1 and different levels of NaCl (1M and 2M NaCl) were added to the plates. For control, just distilled water was applied at the rate of 3 mL/day. Likewise, and an additional set of Petri plates was prepared with same conditions except seed treated with 2 mL sterilized broth medium. The experiment was conducted in a growth chamber with a relative humidity of 60%, a temperature of 24 + 2 °C, and a photoperiod of 14 h per day and 10 h at night. After every 24 h interval, seed germination was assessed for 10 days. Biochemical growth parameter, i.e., chlorophyll content, on fresh weight basis after 15 days of sowing were assessed upon harvesting [33]. To estimate photosynthetic pigments (chlorophyll a, b, and carotenoids), 100 mg fresh wheat seedling leaf samples were crushed in 8 mL acetone (80%) After centrifugation at 4000 rpm for 10 min OD of the supernatant was recorded at 645, 663, and 470 nm by using UV-spectrophotometer [34,35]. Chlorophyll-*a*, chlorophyll-*b*, and carotenoids were estimated by using equation; Chlorophyll a = 1.07 (OD_{663}) − 0.09 (OD_{645}), Chlorophyll b = 1.77 (OD_{645}) − 0.28 (OD_{663}), and Carotenoids = $OD_{470} \times 4$.

Electrolyte leakage of the wheat leaf was assessed by following [36]. Wheat leaves were sliced into 5 mm long pieces and put into test tubes containing 10 mL deionized water. The electrical conductivity of medium (EC1) was determined after the tubes were put on shaking at 30 °C for 4 h. Then all test tubes were autoclaved at 121 °C for 20 min and cooled to 25 °C, and final electrical conductivity (EC2) was recorded. Electrolyte leakage was calculated by using the following formula.

$$\text{Electrolyte leakage} = \text{EC1}/\text{EC2} \times 100 \quad (2)$$

2.7. Statistical Analysis

Whole experimental data were analyzed statistically using one-way analysis of variance (ANOVA) through Statistix 8.1 package. The least significant difference (LSD) test was applied to detect differences among the data set ($p \leq 0.05$). R plot software was used to carry out Principal Component Analysis (PCA) through and Pearson correlation analysis to whole data of the experiment as a single set. For PCA, built-in R function "prcomp" and "ggplot2" library was used.

3. Results

3.1. Biochemical and Molecular Characterization of the Strain PM34

Based on various plant-growth promoting characters and extracellular enzyme activities, the strain PM34 was classified as a PGPR. The strain exhibited nitrogen fixation, potassium, and zinc solubilization activity. Indole acetic acid, siderophore, and ammonia production by the strain PM34 were also positive (Figures S4, S6 and S7). The strain also exhibited positive response for several extracellular enzyme activities including ACC deaminase, pectinase, protease, amylase, catalase, cellulase, and chitinase enzyme (Table 2). The strain PM34 was identified as *Bacillus aryabhattai* on the basis of 16S rRNA gene sequencing and phylogenetic tree showed its relationship with its closest phylogeny (Figure S3). The bacterial strain was submitted to NCBI with the accession number MW316894. PCR was used to amplify genes (*ituC* and *nifH* and *acds*) potentially involved in the biosynthesis of lipopeptides, nitrogenase, and ACC deaminase enzyme, respectively. Amplicons of expected sizes were obtained with the primer pairs designed to detect these genes (Figure S1).

3.2. Abiotic and Biotic Stress Tolerance Assay

Salt stress assay showed that the strain *Bacillus* sp. PM34 has tolerance against both levels (1M-2M NaCl) of salinity stress (Figure 1a). However, the growth of bacterial strain PM34 was dramatically decreased with increase in salt stress. Moreover, the strain PM34 also revealed a significant increase in growth and tolerance at 100 mg/L Ni and 300 levels of Cd (Figure 1c). After incubation for 7 d at ideal temperature, maximum OD (600 nm) of bacterial cells was 1.61 and at 50 °C and 60 °C was 0.8 and 0.7, respectively (Figure 1d). The

results of biocontrol assay showed that the growth of *R. solani* and *F. solani* was significantly minimized with inoculation of antagonistic bacterial strain PM34 in dual culture assay as compared to control (Figure 1b and Figure S5). Maximum inhibition (40.1%) was observed against *R. solani* as compared to control.

Table 2. Morpho-biochemical characteristics of *Bacillus aryabhattai* PM34 (MW316894).

Bacterial Traits	Results
Morphological and colony features	Medium size colony, wrinkled growth, off-white, smooth colonies at 32 °C after 1 d on LB agar.
Bacterial cell traits	Gram-positive, under microscope, showed scattered arrangement of short rod-shaped cells.
Biochemical properties	Positive for atmospheric nitrogen-fixing ability, potassium solubilization, zinc solubilization, IAA production, siderophore, ammonia, ACC deaminase, pectinase, protease, amylase, catalase, cellulase, chitinase, and exopolysaccharides production.

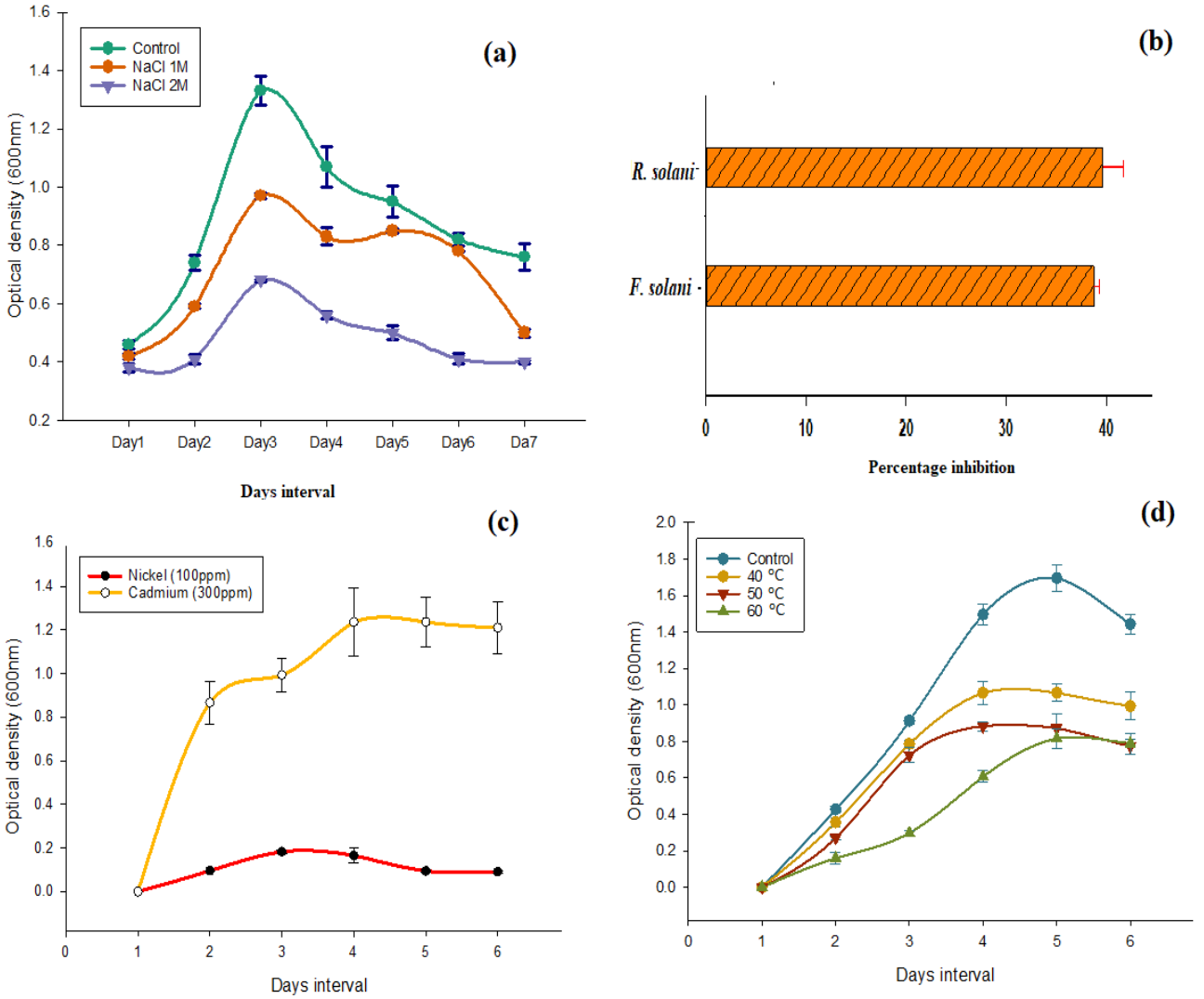

Figure 1. Growth curve analysis of *B. aryabhattai* PM34 at various levels of salt stress (**a**), represents mycelial inhibition of *R. solani* and *F. solani* (**b**), 100 and 300 ppm nickel, and cadmium, respectively (**c**), various levels of heat stress (**d**).

3.3. Wheat Germination and Growth Responses under Salt Stress

Wheat growth and biomass including shoot and root length were substantially influenced with application of the strain PM34 under saline and normal conditions (Table 3). The decrease in growth of wheat seedling was experienced under salinity stress conditions (1.0 M–2.0 M) compared with respective controls. The influence of strain PM34 on growth parameters of wheat was more noticeable than uninoculated control treatment. The application of salt stress decreased the wheat germination significantly ($p < 0.05$) and maximum decrease in germination, i.e., 64% was noted under 2 M NaCl conditions. While the inoculation of strain PM34 enhanced the germination% significantly ($p < 0.05$) by 48% as compared to un-inoculated control under 2 M NaCl level (Table 3). In the same way, root length of wheat seedling was decreased significantly by 96% with 2 M salt. However, the inoculation of strain PM34 enhanced the root length significantly by 80% over un-inoculated control at 2 M NaCl level. The shoot length of wheat seedling was decreased significantly by 91% with 2 M salt. However, the inoculation of strain PM34 enhanced the shot length significantly by 75% over to un-inoculated control at 2 M NaCl level. Fresh and dry biomass of wheat seedling were decreased with both levels of salt stress but the maximum decrease in fresh and dry biomass was observed with 2 M salt stress. On the other hand, bacterial inoculation improved fresh and dry biomass significantly ($p < 0.05$) under normal and both levels of salt stress. Electrolyte leakage was significantly increased under both levels of salt stress and maximum increase (39%) in EL was observed under 2 M NaCl as compared to control. Inoculation of strain PM34 significantly ($p < 0.05$) decreased the membrane damage under both levels of salt stress over un-inoculated control under both levels of salt stress (Figure 2a, Figure S2).

Table 3. Effects of *B. aryabhattai* PM34 on germination %, root length shoot length fresh weight and dry weight of wheat plants grown in Petri plates at two levels of salt stress.

Treatments	Germination %		Root Length (cm)		Shoot Length (cm)		Fresh Weight (g)		Dry Weight (g)	
	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated	Un-inoculated	Inoculated
Control	68.3 ± 2.3^{b}	88.3 ± 6.2^{a}	17.7 ± 0.55^{b}	20.7 ± 0.71^{a}	13.13 ± 0.27^{b}	14.74 ± 0.05^{a}	0.39 ± 0.02^{b}	0.50 ± 0.06^{a}	0.16 ± 0.2^{b}	0.20 ± 0^{a}
NaCl 1M	$40. \pm 4^{c}$	65 ± 4.8^{b}	$3. \pm 0.46^{d}$	5.96 ± 0.57^{c}	1.74 ± 0.07^{e}	7.66 ± 0.69^{c}	0.15 ± 0.01^{d}	0.28 ± 0^{c}	0.07 ± 0^{d}	0.12 ± 0.08^{c}
NaCl 2 M	23.3 ± 2.3^{d}	48.5 ± 4.7^{c}	0.5 ± 0.08^{e}	2.73 ± 0.16^{d}	1.08 ± 0.29^{f}	4.46 ± 0.85^{d}	0.04 ± 0^{e}	0.22 ± 0.01^{c}	0.01 ± 0^{e}	0.09 ± 0

Effects of different treatments of salt on wheat seedling grown in Petri plates. The letters (a–f) in superscripts show significance among inoculated and un-inoculated conditions at $p \leq 0.05$ level. Values are presented as means $\pm$ *SD* (n = 3).

3.4. Chlorophyll Contents

Chlorophyll content (Chlorophyll *a*, *b*, and carotenoids) were significantly ($p < 0.05$) decreased under both levels of applied salt stress. However, the inoculation of strain PM34 significantly ($p < 0.05$) improved the leaf chlorophyll content (Figure 2). Maximum significant increase (28%) in chlorophyll *a* was observed with inoculation of strain PM34 as compared to control without salt stress. As the salt stress increased the chlorophyll content decreased while maximum significant decrease, i.e., 60% in chlorophyll *a* was observed under 2M NaCl stress without inoculation but with inoculation of strain PM34 the chlorophyll *a* was significantly enhanced by 72% as compared to non-inoculated control under 2M NaCl level. In the same way, chlorophyll *b* and carotenoids content were also decreased significantly as the salt stress increased but inoculation of strain PM34 enhanced chlorophyll *b* and carotenoids significantly under normal as well as both levels of salinity.

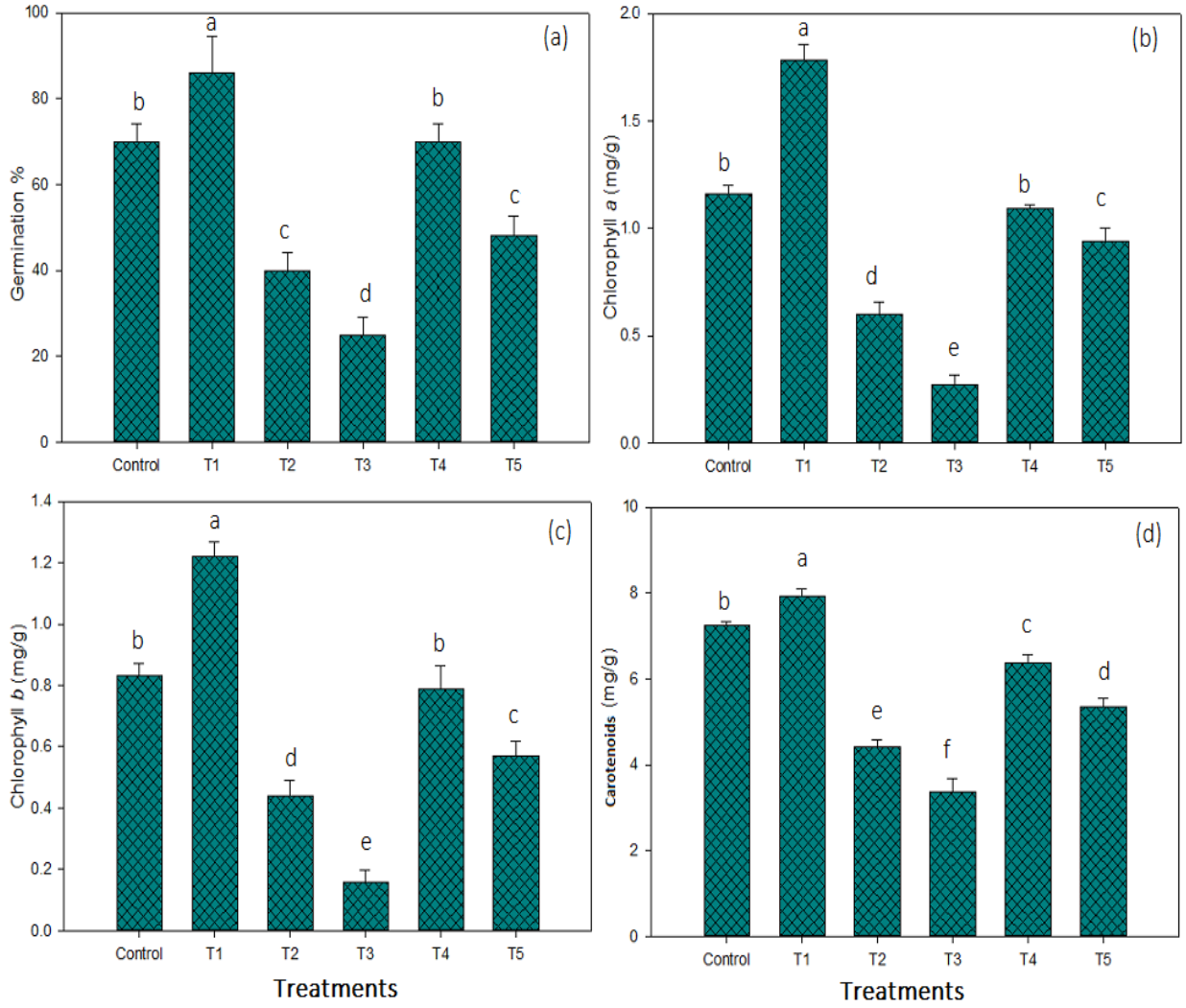

Figure 2. Effects of *B. aryabhattai* PM34 on electrolyte leakage (**a**), chlorophyll *a* (**b**), chlorophyll *b* (**c**), and carotenoids (**d**), of wheat under various levels of salinity stress. Each treatment value is presented as means of three replications (n = 3) with *SE*. Control represents non-inoculated (control), T1 (*B. aryabhattai* PM34), T2 (NaCl 1M), T3 (NaCl 2M), T4 (*B. aryabhattai* PM34 + NaCl 1M), and T5 (*B. aryabhattai* PM34 + 2M NaCl). Treatment means sharing different letter(s) are significantly different at $p < 0.05$ level.

3.5. Principal Component and Pearson Correlation Analysis

The principal component analysis clusters the input and response variables into numerous clusters based on similarity and difference in their correlation and variance. PCA separated the wheat plant's response variables under bacterial strain *Bacillus aryabhattai* PM34 application (Figure 3a). The chlorophyll pigments were grouped, showing their similar increasing response under bacterial strain PM34 inoculation under normal and stress conditions. Biomass parameters root shoot length and fresh and dry weight were also positively correlated. Biplot divided six treatments into separate groups, indicating that the results of these treatments for different plant response attributes differed from each other (Figure 3b). A strong correlation was observed among all studied parameters of wheat seedlings (Figure 4).

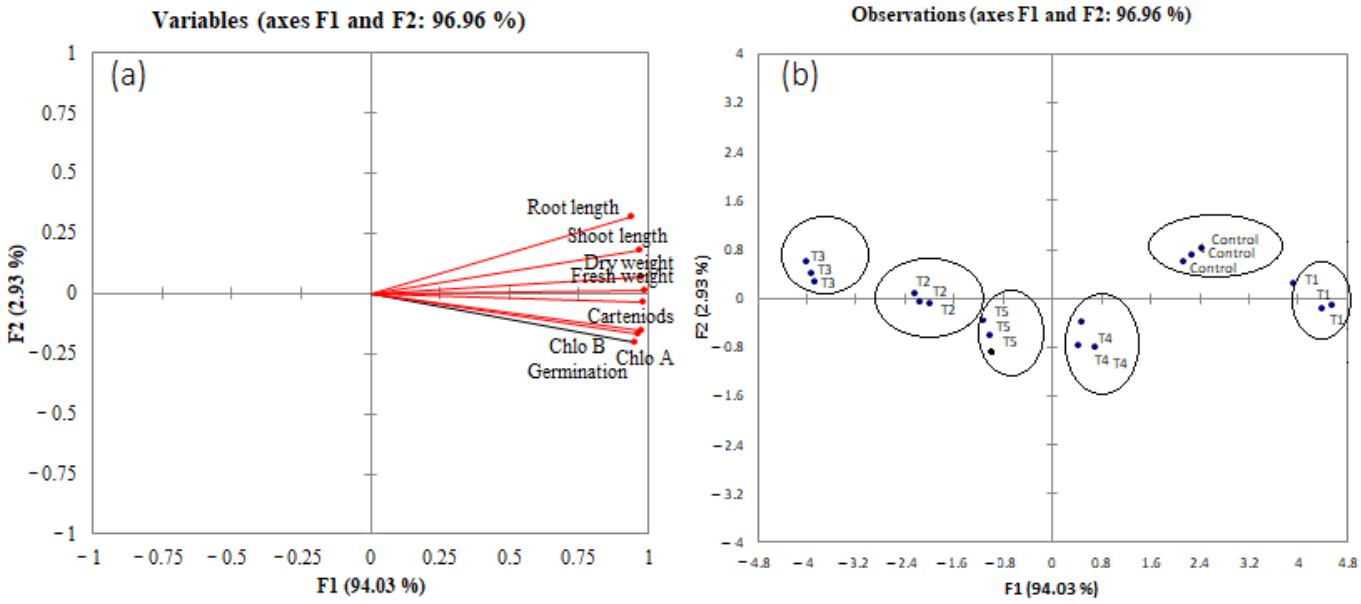

Figure 3. The PCA biplots showing effect of strain PM34 on correlation among various growth variables of wheat seedlings (**a**) and various treatments (**b**) under two levels (1 M and 2 M NaCl) of salt stress.

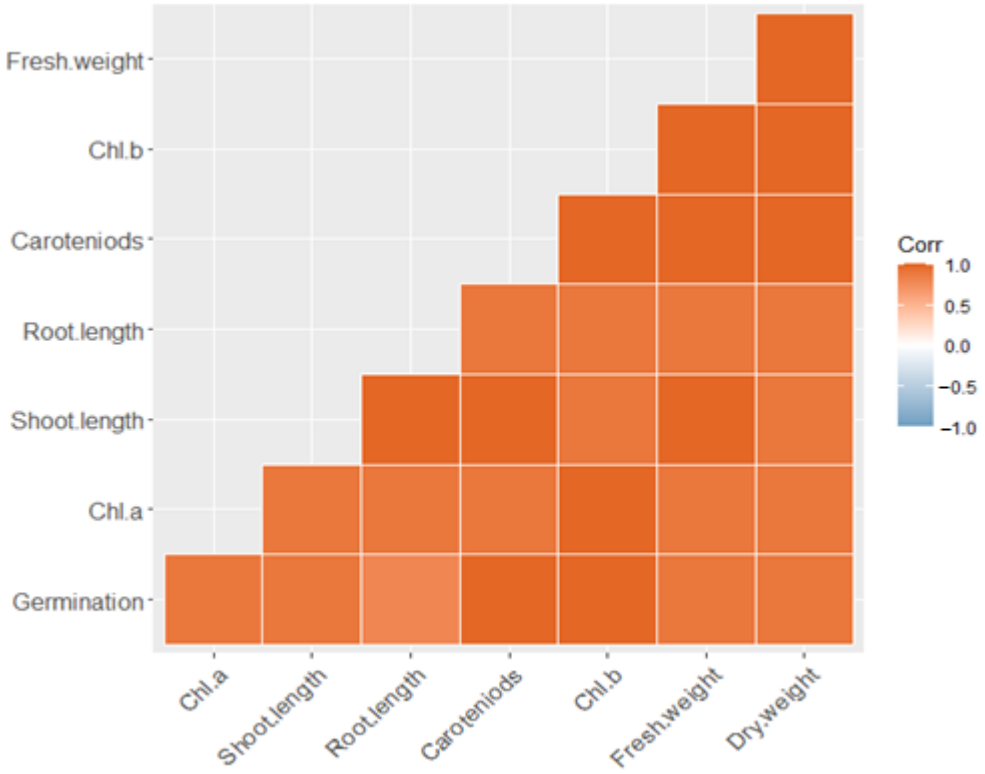

Figure 4. Pearson correlation analysis of applied treatments (strain PM34) on biomass and biochemical attributes of wheat seedling sunder two levels (1 M and 2 M NaCl) of salt stress.

4. Discussion

The selection, screening, and application of multi-stress tolerant bacterial strains would be a key solution to encounter abiotic and biotic stress challenges due to changing climate, low crop yield, and increasing food demand [17,31]. These environment-friendly approaches can also reduce the toxic influences of synthetic fertilizer and pesticide residue

in the food chain [37–40]. In this study, salt (NaCl) stress was applied to in vitro germinating seedlings of wheat and examined the plant growth potential in the presence and absence of *Bacillus aryabhattai* PM34 under salt stress conditions. The bacterial strain *B. aryabhattai* PM34 was equipped with multiple plant growth-promoting (PGP) attributes including nitrogen fixation, potassium, zinc solubilization, indole acetic acid, siderophore, and ammonia production along with various extracellular enzyme activities [17,32,33]. These attributes previously appeared to promote plant growth under normal and stress conditions. Siderophore production, IAA, potassium, and zinc solubilization are beneficial for plant growth under both normal as well stressful environment [41]. Moreover, detection of *ituC* gene responsible for iturin, *nifH* gene responsible for nitrogenase, and *acds* gene responsible for ACC deaminase are well-known traits with the potential to promote plant growth under abiotic and biotic stress conditions [18]. These traits (*ituC, nifH*, and *acds* genes by bacterial strain PM34) are confirmed in this study, and thus these conferred the PGP activity of strain PM34.

The strain PM34 showed significant tolerance at 2 M salt stress. Isolation and characterization of salt tolerant bacterial strains have been well documented [3,4,42]. The growth curve analysis of strain PM34 demonstrates its ability to tolerate high levels of salt stress. The NaCl tolerance of *Bacillus aryabhattai* has been reported previously [42–44]. However, we demonstrated tolerance at high NaCl concentrations than previous reports. In another study at low salt concentrations (80–160 mM) *Kocuria rhizophila* 14ASP and *Cronobacter sakazakii* OF115 inoculation increased photosynthetic pigments in the wheat seedling [4].

In the in vitro experiment, the germination, plant biomass, and physiological parameters including chlorophyll content of germinating wheat seedlings were improved under normal and NaCl stress conditions upon inoculation of strain PM34. It might be due to production of IAA and exopolysaccharides by strain PM34. EPS contain 97% H_2O in the form of polymer matrix, EPS has strong potential to protect seedlings under stressful environment (3). Germination and growth of plant are boosted because of such attributes under stress condition. Moreover, PGPR also enhances not only metabolic activity within seed but also provides cell adhesion, cell-cell signaling, and protection from NaCl stress [25].

When coping with single abiotic stress the main constraint in the agriculture cropping system is the prevalence of multiple stresses (biotic and abiotic), where the resilience of applied bio-inoculants is a significant apprehension [17]. The strain PM34 showed strong antagonistic activity against two fungal strain (*R. solani* and *F. solani*) that shows its potential to endure both abiotic and biotic stresses. The application of multiple-stress tolerant bacteria such as PM34 for dual purpose usage against salinity and fungal stress can play a significant role towards global food security. Results presented by [17] are in line with our results, in which the growth of sugar cane was significantly enhanced with the application of multi stress tolerant bacteria under fugal biotic stress. *B. aryabhattai* PM34 was positive for extracellular enzymes (protease, catalase, amylase, pectinase, cellulase, chitinase) with significant inhibition of both the fungal pathogens (*F. solani* and *R. solani*) in the present study. The strain PM34 might produce numerous secondary metabolites, which have been reported the potential source of fungal mycelial growth restriction. Both diffusible and volatile metabolites have been produced by bacteria which may act against phytopathogenic fungi by many mechanisms including minimizing germination, inhibition, and the lysis of fungal mycelium [45–47]. Furthermore, it has been well documented that plant growth promotion and fungal pathogen control can be achieved by the production of extracellular enzymes (protease, catalase, amylase, pectinase, cellulase, chitinase) by bacteria that can degrade the fungal cell wall [18].

Combined PCA analysis of data including percent germination, plant biomass, and chlorophyll content was carried out which depicted that the overall effect of strain PM34 on plant responses varied from each other and control. Categorization of plant biomass with chlorophyll content in the PCA depicted an increasing response under bacterial treatment. The application of multivariate analysis has acquired considerable significance to find possible trends and relationships among data sets [36,37]. It was also suggested that

under particular situations, the common statistical investigation may not enough to find significance among different treatments and variables but multivariate analysis like PCA analysis separates data sets on the basis of their overall effect to various response variables.

The current investigation opens potential ways for scientists to explore the genetic mechanisms implicated in the induction of salt tolerance by strain PM34 in germinating wheat seedlings. A study on expression profiling of stress-responsive genes in wheat in response to applied bacterial inoculation might be helping to understand the molecular cross-talk between plants and bacterial strains. Treatment with PGPR promotes different molecular mechanisms in plants to safeguard them not only from salt stress but also from soil-borne diseases and increase plant growth and development.

5. Conclusions

Inoculation of *B. aryabhattai* strain PM34 to wheat seedlings under salt stress resulted in a significant reduction of toxic ions, and electrolyte leakage whereas a positive impact on seed germination, biomass, and chlorophyll pigments. The isolated strain PM34 was equipped with various PGP traits including nitrogen fixation, ACC deaminase, potassium, zinc solubilization, and indole acetic acid as well as extracellular enzyme activities. Some of the traits such as *ituC, nifH*, and *acds* were confirmed by the PCR. The strain PM34 showed significant tolerance at 2 M salt stress (NaCl) and inhibited the pathogenic activity significantly by retarding the mycelial growth of *R. solani* and *F. solani*. It could be concluded that in vitro characterization of *B. aryabhattai* PM34 and seedling experiment accomplished its role to improve wheat growth under fungal and salt stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13148030/s1, Figure S1: PCR product showing amplified *iturin* (A), *nifH* (B) and *acds* (C) gene fragment of bacterial strain PM34 on 1.5% agarose electrophoresis. Lanes: M, Molecular mass standards, Figure S2. Effects of strain PM34 on growth of wheat seedlings under the 2molar NaCl stress, Figure S3. The evolutionary history was constructed by Neighbor-Joining method. Bootstrap test—800 replicates, Figure S4. Gram staining results of strain PM34, Figure S5. Mycelial inhibition of *F. solani* (A) and *R. solani* (B) in dual culture experiment by strain PM34, Figure S6. Siderophore production by strain PM34, Figure S7. Zinc solubilization by strain PM34.

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