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RESEARCH ARTICLE

REVISED Using halotolerant *Azotobacter chroococcum* W4ii from technosoils to mitigate wheat salt stress

[version 4; peer review: 2 approved, 1 not approved]

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Abstract

Background

Technosoils in Inowrocław, central Poland, are impacted by human activities and exhibit high salinity (ECe up to 70 dS/m) due to a soda lime repository. These saline environments pose challenges to plant growth and soil health. However, they also offer an opportunity for the evolution of microorganisms adapted to such conditions, including plant growth-promoting rhizospheric (PGPR) bacteria. The hypothesis tested here was that introducing PGPR bacteria from these environments could boost degraded soil performance, leading to better plant biomass and improved pathogen defense.

Methods

Azotobacter chroococcum W4ii was isolated from the rhizosphere of wheat (*Triticum aestivum* L.) for its plant growth properties on wheat plants under salt stress.

Results

Wheat seeds co-inoculated with *A. chroococcum* W4ii under 200 mM salt stress showed significant improvement in various growth parameters such as seeds germination (by 130%), shoot biomass (15%), chlorophyll *b* content (40%) compared to un-inoculated ones.

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Bacterial inoculation decreased the level of malondialdehyde (MDA) by 55.5% ($P<0.001$), whereas it elevated the antioxidative enzymatic activities of peroxidase (POD) by 33.69% ($P<0.001$). The test isolate also significantly ($P<0.05$) enhanced the level of defense enzymes like β-1,3-glucanase, which can protect plants from infection by pathogens. The bacterium could also successfully colonize the wheat plants.

Conclusions

These results indicate that *A. chroococcum* isolated from the technosoil has the potential to promote wheat growth under salt stress and can be further used as a bioinoculant in the salt affected agricultural fields.

Keywords
PGPR, Azotobacter chroococcum, Salt stress, Technosoil, Triticum aestivum L.

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REVISED **Amendments from Version 3**

The abstract has been modified in accordance with the reviewer's comments.

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Introduction

Wheat (*Triticum aestivum* L.) is the most traded and planted staple food crop globally, covering 216 million hectares with total production of 765 million tons worldwide (FAOSTAT, 2019). However, due to climate change and various anthropogenic activities, salinity is increasing and subsequently affecting 20% of global cultivable land (Saddiq *et al.*, 2021). The soil salinity is also distressing the wheat production leading to the yield loss up to 60% (Dadshani *et al.*, 2019). In the European Union (especially Mediterranean countries) alone, one million hectares are affected by soil salinity (Machado & Serralheiro, 2017).

Salt stress causes nutrient deficiency due to excessive accumulation of salt ions that inhibits the absorption of other nutrient ions by plants. Soil salinization leads to various other secondary effects like hyperosmotic stress induced water deficit and increased production of reactive oxygen species (ROS) (Ji *et al.*, 2022).

However, application of plant growth-promoting rhizobacteria (PGPR) improves plant growth under salinity stress conditions (Bharti *et al.*, 2016; Ji *et al.*, 2022; Singh & Jha, 2017; Upadhyay & Singh, 2015). PGPR as bio-inoculants *e.g.*, *Agrobacterium*, *Azospirillum*, *Bacillus,* and *Pseudomonas* species are well established, economically feasible, and environment friendly approach to reclaim salinity affected land for enhanced crop yield (Upadhyay & Singh, 2015). Agronomic yields in response to PGPR have been increased due to the production of phytohormones (indole-3-acetic acid, gibberellic acid, zeatin, ethylene, abscisic acid), P solubilization, N fixation, siderophore and ACC deaminase enzyme production (Abbas *et al.,* 2019). PGPR also promote plant growth by altering the selectivity of Na^+, K^+, and Ca^{2+} and sustaining a higher K^+/Na^+ ratio in plants under salt stress (Hamdia *et al.*, 2004). Improvement in wheat cultivation in saline areas have been reported to be facilitated by the application of PGPR (Desoky *et al.*, 2020; Nadeem *et al.*, 2013; Nawaz *et al.*, 2020).

Further, technosoils present in Inowrocław, central Poland harbor varied microbial communities. The technosoil represents a unique extreme environment that is located in the proximity of soda lime repository ponds of CIECH Soda Polska S.A (Hulisz *et al.*, 2018). The leaching of chloride salts (mainly NaCl and $CaCl_2$) into groundwater makes the soil extremely saline (ECe up to 60 dS/m) and alkaline (pH up to 8) (Gołub & Piekutin, 2020; Hulisz & Piernik, 2013). Microorganisms naturally adapted to such extreme habitats have the potential to cope with various stresses like salinity.

Moreover, *Azotobacter* genus is a free-living, aerobic nitrogen-fixing, bacteria and the first identified species in this genus was *A. chroococcum* (Robson *et al.*, 2015). *A. chroococcum* has been used widely as a PGPR to stimulate the growth of various plant varieties under different soil types and climatic conditions (Romero-Perdomo *et al.*, 2017). Regulation EU 2019/1009 has recognized *Azotobacter* as one of the three bacterial genera that can be used as microbial plant biostimulant/biofertilizer (Kerečki *et al.*, 2022). *A. chroococcum* according to different studies was found to reduce N fertilization in cotton (Romero-Perdomo *et al.*, 2017), reduce mean germination time in sugar beet (Kerečki *et al.*, 2022) and Hopbush Shrub (Yousefi *et al.*, 2017), mitigate metal stress in maize (Rizvi & Khan, 2018), increase nitrogen content of *Adathoda vasica* (Naik *et al.*, 2007) and enhance adaptation of tomato plants under salt stress and N stress (Van Oosten *et al.*, 2018). Other studies have suggested that it ameliorated the negative impact of NaCl in crops like maize and wheat (Allam *et al.*, 2018; ÇAM *et al.*, 2022; Silini *et al.*, 2016).

Therefore, in the present study, an attempt was made to further explore the potential of salt-tolerant nitrogen fixing *A. chroococcum* W4ii isolated from the rhizosphere of wheat grown in the technosoils for its plant growth promoting traits in the wheat plant grown in soil under saline stress. For this, a preparation of W4ii isolate with skimmed milk was prepared and treated with the wheat plant growing under salt stress. Different biochemical and growth parameters of wheat plants were also determined to test its efficacy to confer salt stress tolerance.

Methods

Isolation of bacterial strain

The bacterial strain was isolated from the rhizosphere of wheat grown in the technosoils in Inowrocław, central Poland. A soil sample of 5 g was added to 45 mL of nitrogen free JMV (mannitol as the carbon source) for the enrichment of diazotrophs (Baldani *et al.*, 2014). After seven days, 100 µl of the above prepared soil suspension was again inoculated into vials containing 5 mL of fresh semisolid JMV media and kept at 26°C. Diffuse subsurface growth pellicles appeared in vials after five days, then a loop full of culture from these vials was streaked onto the nitrogen-free solid medium. Single, separated colonies growing on these plates were again streaked on the solid JMV agar plate.

Subsequently, colony PCR with primer set Pol F-Pol R (Poly *et al.*, 2001) for *nif*H gene was performed. Positive strains for *nif*H were selected for further identification using 16S rRNA gene amplification.

Identification of the strain

Total genomic DNA was extracted from the strain using the Gene MATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland; cat. no E3580) following the instructions given by the manufacturer. The 16S rRNA gene was amplified, sequenced, and identified using the EzBioCloud database as described in our previous study (Kalwasińska *et al.*, 2017).

Test for plant growth promoting features and stress tolerance of *A. chroococcum* W4ii

The isolated strain was evaluated for its ability to fix N_2 by the acetylene reduction assay (ARA) (Beauchamp *et al.*, 1991). Briefly, each flask was closed with a Suba (Sigma-Aldrich, USA) and 10% (v/v) of the air was replaced by acetylene. The flasks were incubated for 30 min at room temperature and ethylene concentration was determined by injecting 0.1 mL of air sample in a gas chromatograph (Perkin-Elmer, Montreal, QC, Canada) equipped with a flame ionization detector (FID) and a 0.5 mm, 30 m RTQ-PLOT column (Restek, Bellefonte, PA, USA). The presence of ACC deaminase enzyme in the strain was tested by measuring the amount of α-ketobutyrate production (a cleavage product of ACC) spectrophotometrically (SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) at 540 nm using the standard curve of α-ketobutyrate (Ali *et al.*, 2014). A test of phosphate solubilization was performed in Pikovskaya (PVK) media agar plates supplemented with insoluble tricalcium phosphate as the phosphate source (Sharon *et al.*, 2016). The spot cultured plate was incubated at 26°C for seven days and colonies with a clear halo were marked positive for phosphates solubilization. For the IAA production test, isolated strains were propagated in DF salts minimal media supplemented with 5 mM of L-tryptophan. The supernatant of bacterial culture was then mixed with Salkowski's reagent after 42 h of incubation. The absorbance at 535 nm was measured spectrophotometrically using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) and the concentration of IAA in each culture medium was determined by comparison with a standard curve (Patten & Glick, 2002). Test for Siderophore production was evaluated on chrome azurole S-agar (CAS-agar) plates and observed for the formation of a color zone around the point inoculated colony (Schwyn & Neilands, 1987). Assay for ammonia production was tested using Ammonia Assay Kit purchased from Sigma-Aldrich (cat. no MAK310).

The tolerance of the isolate toward abiotic stressors like pH and salinity was studied. Salt tolerance (0, 1, 2 and 4 % NaCl, w/v) and pH (6–9) tolerance were tested on liquid JMV media. For both salt and pH studies, 50 µl of freshly grown culture (0.5 OD) was added to the media with different pH and salt concentration. Optical density (OD) was then determined on the seventh day with the help of densitometer DEN-18 (Biosan, Rīga, Latvia) to test the salt and pH tolerance.

Effect of *A. chroococcum* W4ii on plant growth with and without salt treatment

Preliminary pot experiment was performed to check the efficiency of the strain on wheat plant growth in the presence and absence of salt stress. For this, seeds were treated with bacterial inoculum of 0.3 OD to avoid additional stress to the seeds. The seeds were then surface sterilized by treating with 70% ethanol followed by 2% sodium hypochlorite (NaOCl) solution for 3 min and subsequently washed using sterile water to remove all traces of sodium hypochlorite. The surface-sterilized seeds of wheat were kept in the bacterial suspension for 1 h. Surface sterilized seeds were treated with 0.03 M $MgSO_4$ instead of bacterial suspension served as control. A total of 20 bacterized seeds were sown in each plastic pot (8 cm in height, 11 cm in diameter) filled with sterilized sand and vermiculite mixture grown in a controlled environment of plant growth chamber (13:11 photoperiods for 15 days at 15 ± 2°C, 1 h of dawn and dusk time and 65% light intensity *i.e.*, 1,040 klux m^{-2}). For providing salt stress and nutrient, 200 mM of NaCl was added to the Hoagland medium. For comparative analysis, a set of control plants with 0 mM NaCl was taken. On the fifth day, again bacterial suspension of approximately 10^8 CFU/mL was applied to the pots. Plants were watered every day after germination. The seeds were also checked at 150 mM salt concentration, where the plant growth was similar to the growth of plants under 200 mM salt concentration. Therefore, 200 mM salt concentration was selected for further experiments. Pots were arranged in a completely randomized block design with three replications in each treatment.

For measuring growth (root/shoot length) and biomass (fresh/dry weight), five randomly selected plants from each replicate were used. The percentage of germination was also calculated according to the formula: *Percentage of germination* = $(\sum n \div N) \times 100$ (Fatemeh *et al.*, 2014). To estimate the chlorophyll content, fresh leaf samples of 0.1 g were homogenized with 80% acetone (10) and centrifuged at 5,000 rpm for 10 min at 4°C. The absorbance of collected supernatant was read spectrophotometrically at λ = 645 and λ = 663 nm using SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). The same was calculated as follows (Liang *et al.*, 2017):

Total chlorophyll (µg/mL) = 20.2 (A_{645}) + 8.02 (A_{663})

Chlorophyll *a* (µg/mL) = 12.7 (A_{663}) – 2.69 (A_{645})

Chlorophyll *b* (µg/mL) = 22.9 (A_{645}) – 4.68 (A_{663})

Effect of *A. chroococcum* W4ii on plant growth under salinity stress

In the pot experiment mentioned above, which used sand as the growing medium, we found no significant difference in overall plant growth, seed germination, plant weight (fresh and dry), or chlorophyll between bacteria-treated and non-treated plants in a 0 mM salt concentration. However, we observed such differences ($p<0.05$) under salinity conditions. Therefore, we performed further pot experiments in soil using the *A. chroococcum* W4ii formulation only in a 200 mM salt concentration.

A. chroococcum W4ii inoculum preparation. The strain was grown on solid JMV medium (g L^{-1}) mannitol, 5.0; K_2HPO_4, 0.6; KH_2PO_4, 1.8; $MgSO_4$.7H2O, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.02; Micronutrient solution ($CuSO_4.5H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.12; H_3BO_3, 1.40; $Na_2MoO_4.2H_2O$, 1.0; $MnSO_4$. H_2O, 1.175. Complete volume to 1,000 mL with distilled water), 2 mL; bromothymol blue (5 g L^{-1} in 0.2 N KOH), 2 mL; FeEDTA (16.4 g L^{-1}), 4 mL; vitamin solution (biotin, 10 mg/100 mL), 1 mL. pH was adjusted with KOH. Agar 17 g L^{-1} was added. Total 10 Petri dishes (200 mm diameter) were used to grow the bacteria. After one week, the bacterial cell mass was collected and mixed with 10% skimmed milk. The bacterial cell

mixture with skimmed milk was then lyophilized to get the powered formulation to be applied in the soil. It was kept at 4°C for further use. Using a carrier-based solid bacterial formulation with skimmed milk instead of a liquid formulation can have several advantages. Skimmed milk is a rich source of nutrients, including proteins, vitamins, and minerals, which can help support the viability and survival of bacterial cells. When used as a carrier, skimmed milk provides essential nutrients for the bacteria, increasing their chances of remaining viable and active for a longer time. Skimmed milk-based solid formulations can enhance the adhesion of bacterial cells to surfaces, such as seeds or plant roots. Using solid formulations reduces the risk of contamination compared to liquid formulations.

A. chroococcum W4ii formulation application on wheat plants. Soil samples (0–20 cm) were collected from an arable field in Inowrocław (coordinates: 52.760456, 18.240217) on September 3rd, 2021. Upon arrival at the laboratory, the soil underwent surface-drying and sieving through a 2-mm mesh to remove plant debris, including root systems and straw. Subsequently, the soil was analyzed to assess its physicochemical properties (Table 1). After the analysis, it was transferred into 20-liter plastic containers and stored in a cold environment at 4°C until the pot experiment was conducted.

The soil analyses were conducted at the Laboratory of Environmental Analysis, Faculty of Earth Sciences and Spatial Management, NCU in Toruń. The electrical conductivity (ECe) was determined in saturated paste extracts using the conductometric method. Soil pH was measured in water (soil to water ratio 1:2.5) through the potentiometric method. The CaCO3 concentration was determined using the Scheibler method (Bauer *et al.*, 1972), while total organic carbon (TOC) and total nitrogen (TN) content were analyzed using a Vario Macro Cube CHN/CHNS microanalyzer (Elementar, Langenselbold, Germany).

The soil used for the study was autoclaved at 121°C for 1 h for three consecutive days to kill any microbial presence. Sterility of the soil was checked by standard serial dilution method. Then, 200 mM of salt was added to each pot without Hoagland medium. Seeds were surface sterilized as described above. A total of 25 seeds were sown in each pot. The prepared *A. chroococcum* W4ii formulation was dissolved in water and approximately 10^8 CFU/mL was added to each pot during sowing and after five days of growth. Only water was added to pot for control treatment. Plants were sprinkled with water every day after germination. The pots were kept in the plant growth chamber with same parameter settings as aforementioned. Pots were arranged in completely randomized block design with three replications in each treatment.

Table 1. Physicochemical properties of soil used for the study.

Compound	Value
Soil classification (WRB)	Mollic *Gleysols*
ECe (dS/m)	0.98
pH (H_2O 1:5)	7.6
TOC (%)	1.7
TN (%)	0.19
C:N	9.0
$CaCO_3$ (%)	2.7

EC_e – electrical conductivity, TOC – total organic carbon, TN – total nitrogen

After 15 days, plants were harvested for other biochemical analysis. Following uprooting, the roots were completely entangled to each other and couldn't be separated. So, fresh and dry weight was measured for whole root system from each replicate. For measuring the biomass (fresh and dry) of shoots, 10 shoots from each replicate (pot) were measured.

Biochemical analysis of plant

Lipid peroxidation. The level of lipid peroxidation was calculated by measuring the malondialdehyde (MDA) content formed through thiobarbituric acid reaction using an MDA assay kit (cat. no. MAK085) purchased from Merck (Darmstadt, Germany). For the assay, 10 mg of leaves were taken for each replicate. The MDA-TBA adduct formed was measured fluorometrically at fluorescence intensity (λ_{ex}=532/λ_{em}=553 nm). The calculations were done according to the kit's specifications.

Total soluble sugar (TSS). Total soluble sugar (TSS) in control and stressed wheat leaf samples were determined according to DuBois *et al.* (1956) with some modifications. About 100 mg of fresh leaves were homogenized in 5 mL of 80% ethanol and incubated in a water bath at 80°C for 30 min. After incubation, 0.5 mL of extract was mixed with 0.5 mL of 5% phenol and 1.5 mL of 95% H_2SO_4 and further incubated in the dark for 15 min. Absorbance was then measured at 490 nm using the SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

Proline. Proline content in the leaves was determined according to the standard protocol (Bates *et al.*, 1973) with minor modifications. Fresh leaves (0.1 g) were homogenized in 1.2 mL of 3% (w/v) sulfosalicylic acid and centrifuged at 13,000 rpm for 10 min at 4°C. Resulting supernatant of 500 µL was mixed with 500 µL water and 1 mL of 2% ninhydrin. The mixture was then boiled for 30 min at 100°C. After cooling, 2 mL of toluene was added to the mixture and upper aqueous phase was used for taking absorbance at 520 nm in a spectrophotometer (SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA)). The proline content was estimated by comparing with a standard curve of L-proline as standard.

Antioxidant assay

The peroxidase (POD) activity in the extract was determined by the method of Kar & Mishra (1976) with modifications. Plant leaves (0.5 g) were extracted in the buffer containing

5 mL of 50 mM phosphate buffer (pH 7.0) added with 1% polyvinylpyrrolidone (PVPP). The crude extract was centrifuged at 10,000 g for 15 min at 4°C, and the obtained supernatant was used for testing (POD) activity. Then, 3 mL of the assay mixture for the peroxidase activity comprised: 0.1 M phosphate buffer (pH 6), 0.027% (w/w) hydrogen peroxide, 0.5% (w/v) Pyrogallol, and 0.1 mL of enzyme extract. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm. In total, 100 mL of distilled water was used as blank instead of enzyme extract.

The activity was calculated according to the formula below:

Units mL^{-1} enzyme= (A_{420} Test - A_{420} Blank)(3)(df) / (12) (0.1)

Where,

3 = Volume (in milliliters) of assay; df = Dilution factor; 12 = Extinction coefficient of 1 mg/mL of Purpurogallin at 420 nm; 0.1 = Volume (in milliliters) of crude enzyme used.

Units mg^{-1} solid = units mL^{-1} enzyme / mg solid mL^{-1} enzyme.

β-1, 3-glucanase enzyme assay

The β-1, 3-glucanase activity was determined according to Wu *et al.* (2018) by using the spectrophotometric method. The wheat roots were placed in sterile distilled water and 10-fold dilution was prepared from that. Subsequently, the roots were shaken for 20 min at 150 rpm. The supernatant of 500 µl was mixed with 500 µl of laminarin 0.5% (v/w) in 100 mM sodium acetate buffer (pH 5.5). The reaction was carried out at 50°C for 60 min and then terminated by heating for 5 min at 100°C. Next, 2 mL of dinitrosalicylate (DNS) 1% (v/w) was added to the reaction and boiled for 10 min. After the samples were cooled, the sugar concentration was measured spectrophotometrically on SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) at 540 nm against a standard curve containing glucose as a standard (Sigma-Aldrich, USA). One unit of β-1, 3-glucanase activity (U) was defined as the amount of glucose released and expressed in µmol/mL/h.

Root colonization efficiency of *A. chroococcum* W4ii

Root colonization of inoculated bacterium was determined after 15 days of plant growth using serial dilution plating technique on JMV-agar medium and the number of viable cells was estimated as colony forming units (CFU). For this, 100 mg of roots from both control and treated plants were washed (6–7 times) and vortexed in N-saline with glass beads.

As *A. chroococcum* W4ii has discrete morphology on the JMV nutrient plate with yellow color and black pigmentation, it was easily identifiable. But for the confirmation, random colonies with the mentioned morphology were further tested for *nif*H gene amplification.

Statistical analysis

The experiment was conducted in completely randomized designs. The difference between each treatment and control group was analyzed by unpaired t-test or Mann-Whitney test using PAST statistical software package (version 4.03) (RRID: SCR_019129). The difference was considered significant if $P \leq 0.05$.

Results

Identification, plant growth promotion properties and stress tolerance of the isolate W4ii

The isolate showed 99.93 16S rDNA similarity (%) with *A. chroococcum* IAM 12666^T (NCBI GenBank, accession number OL348496; Binod Kumar & Kalwasińska, 2021).

The strain was *nif*H gene positive, showed nitrogenase activity in the acetylene reduction assay, and produced ammonia and indole acetic acid (IAA). It was also salt (up to 3%) and pH (up to 9) tolerant. It was negative for ACC deaminase activity, phosphate solubilization and siderophore production.

Growth promotion effect of *A. chroococcum* W4ii on wheat plants under saline and non-saline conditions

In the preliminary study of the pot experiment with sand, no significant difference was found in the growth between control and bacterial inoculated wheat plants grown without any salt stress (Figure 1 and Figure 2) (Kalwasińska, 2023).

In 200 mM salt stress in soil, a significant increase at $P < 0.05$ was there in both root and shoot length in the bacteria treated plant in comparison to the control (Figure 3).

Also, there was a significant increase ($P < 0.05$) in the shoot biomass (dry) treated with bacteria under salt stress (Figure 4). However, there was no improvement in the root biomass under both 0 mM and 200 mM salt treatment when inoculated with bacteria. The percentage of germination was also significantly higher ($P < 0.05$) in the pots treated with the isolate compared to the non-treated ones provided with 200 mM salt concentration (Figure 5). Then again, in the soil without salt; there was no difference in the percentage of germination between control and bacteria treated pots. Also, there was a significant ($P < 0.05$) increase in chlorophyll *b* content in the bacteria treated plants compared to control plants (Figure 6).

Efficiency of *A. chroococcum* W4ii formulation on wheat plant grown in soil under salinity stress

The pot experiments in the sterile sand clearly showed that there was no effect of *A. chroococcum* W4ii on wheat plant growth without salinity stress. It could only protect and promote the growth of plants under saline conditions. Therefore, further experiments were done in the soil environment provided with the salinity stress of 200 mM NaCl. In the given stress, the isolate significantly ($P<0.05$) increased the shoot biomass (both fresh and dry) by one fold approximately (Figure 7).

Inoculation with *A. chroococcum* W4ii significantly reduced the MDA content by 55.5% ($P<0.001$) under the salt stress condition (Figure 8).

However, there was no significant difference in the proline and TSS content after bacterial inoculation compared to the control (Figure 9 and Figure 10).

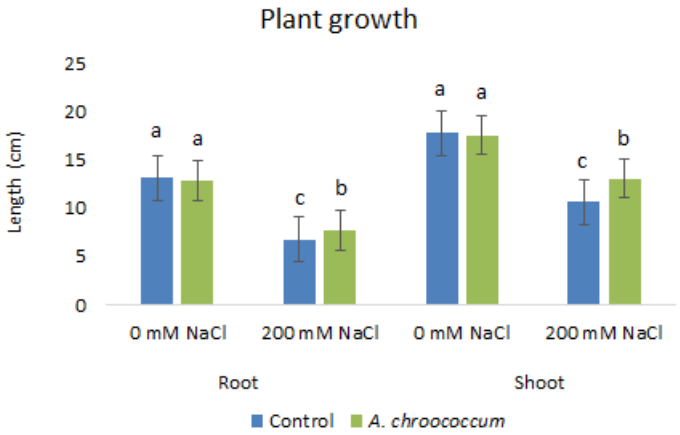

Figure 1. Effect of salinity on plant growth in the sand. Each data represent the mean ± SD of triplicate sets of five measurements (n = 15). Different letters on the bar in each column represent the significant difference.

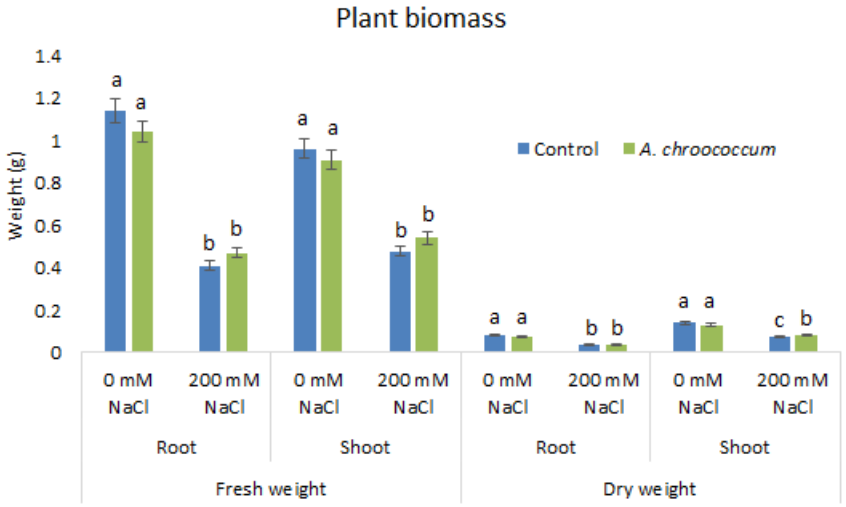

Figure 2. Effect of salinity on plant biomass in the sand. Each data represent the mean ± SD of five plants in triplicate sets (n = 3). Different letters on the bar in each column represent the significant difference.

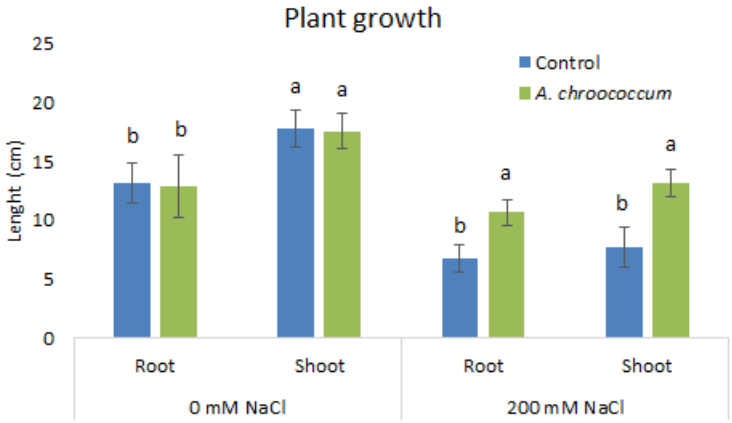

Figure 3. Effect of inoculation of *A. chroococcum* W4ii on plant growth in the sand under 0 mM NaCl and 200 mM NaCl. Each data represent the mean ± SD (n = 15). Different letters on the bar in each column represent the significant difference (P<0.05).

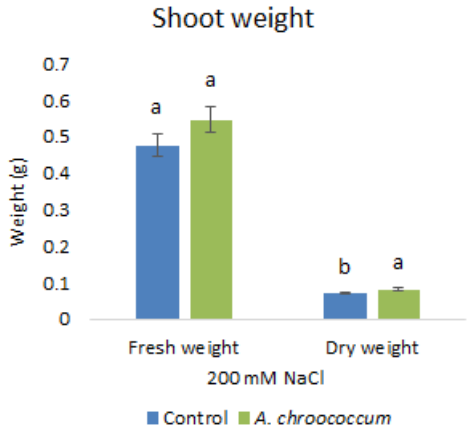

Figure 4. Effect of inoculation of *A. chroococcum* W4ii on plant shoot weight in the sand under 200 mM NaCl. Each data represent the mean ± SD of 5 plants in triplicate sets (n = 3). Different letters on the bar in each column represent the significant difference (P<0.05).

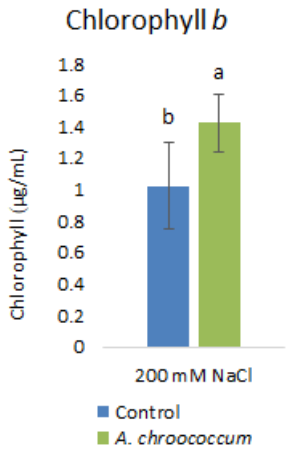

Figure 6. Effect of inoculation of *A. chroococcum* W4ii on plant chlorophyll *b* under 200 mM NaCl in the sand. Each data represent the mean ± SD (n = 6). Different letters on the bar in each column represent the significant difference (P<0.05).

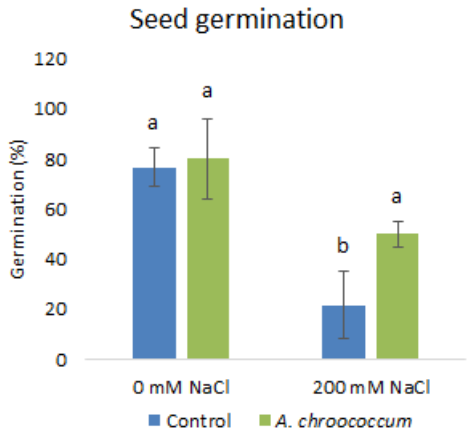

Figure 5. Seed germination in the sand. Each data represent the mean ± SD (n = 3). Different letters on the bar in each column represent the significant difference (P<0.05).

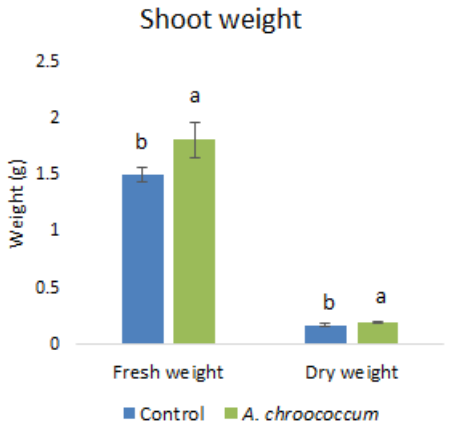

Figure 7. Effect of inoculation of *A. chroococcum* W4ii on plant shoot weight under 200 mM NaCl in soil. Each data represent the mean ± SD of 10 plants in triplicate sets (n = 3). Different letters on the bar in each column represent the significant difference.

Again, inoculation with the isolate significantly (P<0.001) increased the antioxidative activities *i.e.*, peroxidase activity by 33.69% to alleviate the salinity induced free radical damages (Figure 11). In addition to that, there was a two-fold (P<0.05) increase in the defense enzymes *i.e.*, glucanase in the roots of wheat plants treated with the W4ii compared to the control ones (Figure 12).

Root colonization

After 15 days of plant growth, the associated *A. chroococcum* W4ii was found in a range of 10^5 CFU/g of the root. No bacterial colonies were recovered from uninoculated control plants. The *nif*H gene amplification also confirmed that the bacterium was N_2-fixer and indicated that the bacterium had successfully colonized the plant's root system.

Discussion

In the present work, PGPR activity of *A. chroococcum* W4ii and its effect on wheat plant under salinity stress was studied in detail. The bacterium was isolated from the rhizosphere of wheat plant grown in technogenic soil affected by soda industry and thus had inherent resistance to harsh environments. The Polish winter wheat variety (Ostroga) was chosen for the experiments because it has good grain quality, high cropping power and frost resistance (Danko, 2010). Since, the wheat variety is frost resistant, it could be grown very well in the

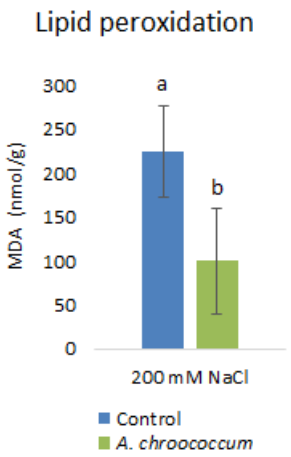

Figure 8. Effect of inoculation of *A. chroococcum* W4ii on plant MDA content under 200 mM NaCl in soil. Each data represent the median ± IQR (n = 9). Different letters on the bar in each column represent the significant difference (P<0.05).

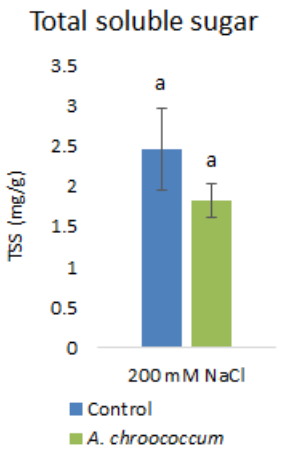

Figure 10. Effect of inoculation of *A. chroococcum* W4ii on plant total soluble sugar (TSS) under 200 mM NaCl in soil. Each data represent the mean ± SD of triplicate sets (n = 3). The same letters on the bar in each column represent no significant difference.

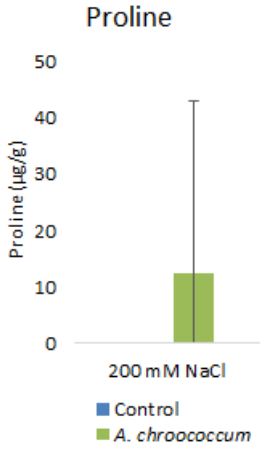

Figure 9. Effect of inoculation of *A. chroococcum* on plant proline content under 200 mM NaCl in soil. Each data represent the median ± IQR of triplicate sets (n = 3).

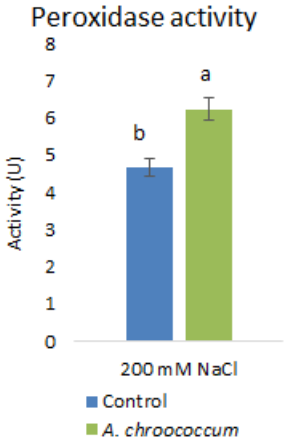

Figure 11. Effect of inoculation of *A. chroococcum* W4ii on plant's peroxidase activity under 200 mM NaCl in soil. Each data represent the mean ± SD (n = 9). Different letters on the bar in each column represent the significant difference (P<0.05).

reclaimed or salt affected soils with the PGPR that can help it to better adapt in saline fields as well.

Our study showed that the isolated PGPR did not have any significant effect on the wheat variety in non-saline conditions. Another reason may be the Na^+-dependent growth of *A. chroococcum*, which has been described for several strains including ATCC 7493 and the type strain ATCC 9043 (Robson *et al.*, 2015). Similarly, a detailed study by Page (1986) concluded that it is important to determine the geographic distribution of Na^+-dependent strains of *A. chroococcum* if they are associated to particular soil types and aware of the Na^+ requirements of the strain if they are unsuited to soil conditions (*i.e.*, the soil has low exchangeable Na^+). Another investigation also revealed that *A. chroococcum* AZ6 did not show any improvement in plant length in non-saline conditions for Waha (*Triticum durum* Waha *lcv*) wheat variety (Silini *et al.*, 2016). Thus, both Na^+-dependency of strains and wheat variety could affect growth promotion in non-saline soil.

However, the wheat variety was not salt resistant as the results from pot trials with sand indicated that the growth of wheat was seriously affected by salinity stress. All the growth parameters of plants *i.e.*, shoot length, root length, shoot (fresh and dry) and root (fresh and dry) weight significantly decreased under 200 mM salt stress. But after treatment with salt-tolerant *A. chroococcum* W4ii, the growth parameters of wheat were

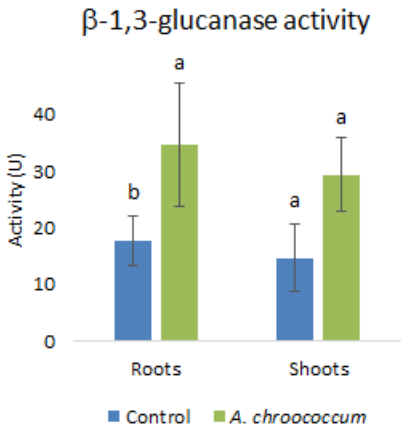

Figure 12. Effect of inoculation of *A. chroococcum* W4ii on root and shoot of plant's β-1,3-glucanase activity under 200 mM NaCl in soil. Each data represent the mean ± SD (n = 3). Different letters on the bar in each column represent the significant difference (P<0.05).

significantly improved under salt stress. It showed consistent results in the improvement of plant length (both root and shoot) and shoot biomass (both fresh and dry) in both sand and soil.

Notably, the fresh and dry weight of the shoot increased after bacterial treatment and there was no effect on the root biomass. In general, the root/shoot ratio is proportional to nutrient supply/fertilization, with a greater ratio at low nutrient supply according to the concept of functional equilibrium. This theory was further approved by Nobel *et al.* (1989) with their study on *Agave lechuguilla*, where N application ha^{-1} increased the shoot dry weight with no effect on root dry weight and reduced the root/shoot ratio. Similarly, Bonifas *et al.* (2005), conferred that the root: shoot ratio decreased for both corn and velvetleaf as a result of increased N supply. Yet again, *A. chroococcum* is known to reduce N-fertilization in many plant species *e.g.*, in *Adathoda vasica* Nees. (Naik *et al.*, 2007), cotton (Romero-Perdomo *et al.*, 2017), maize (Meshram & Shende, 1982) and tomato (Van Oosten *et al.*, 2018). Therefore, it can be interpreted that the isolate W4ii also has the capability of providing nitrogen to the plant and subsequently reducing N-fertilization.

The bacterium not only improved the growth parameters but also helped in the germination of seeds. The percentage of germination was significantly higher (by 130%) in the pots treated with bacteria compared to the control ones. The isolate induced greater germination possibly due to the ability of PGPR to synthesize hormones such as indole acetic acid and gibberellic acid that regulate cell division favoring seed germination (Pérez-García *et al.*, 2023). W4ii also showed the production of IAA that helped in the germination process of seeds. Previous studies conducted by Yousefi *et al.*, 2017 also revealed that under saline conditions (at 20 and 50 dS/m), *A. chroococcum* treatment led to improved germination rate and percentage in hopbush seeds compared to the control. Yet again, *A. chroococcum* loaded nanofiber improved the germination percentage of green gram seeds in the separate study conducted by Kumuthan *et al.*, 2023.

Of note, only chlorophyll *b* content was significantly increased (approximately 40%) after bacterial treatment in the salt treated plants. But there was no significant effect on total chlorophyll content. This finding was very new to our understanding. However, a detailed study by Biswal *et al.*, 2012 on tobacco plants showed that the increased chlorophyll *b* synthesis led to an increase in the capture of light and enhanced (40–80%) electron transport rates of photosystems I and II at both limiting and saturating light intensities. An increase in the light saturated photosynthetic carbon assimilation, starch content, and dry matter accumulation was also reported. Increased level of chlorophyll *b* additionally showed delayed senescence, leading to a longer period of photosynthesis and biomass production. Therefore, application of isolated *A. chroococcum* W4ii strain as a biostimulant may help plants to have longer photosynthetic periods and improved productivity. At this point, more studies are needed.

Salt stress increases the generation of ROS in plants, which enhances the membrane lipid peroxidation and increases the MDA content. When plants are subjected to salinity, MDA is deposited in tissues to indicate membrane destruction (Mousavi *et al.*, 2022; Parida & Das, 2005). Therefore, leaf MDA content is usually used to estimate plant tolerance to salinity (Katsuhara *et al.*, 2005; Luna *et al.*, 2000). The decrease in MDA content in W4ii inoculated plants indicated that bacterial inoculation protected the plants from the imposed salt stress.

Proline accumulation in response to salt stress protects the cell membrane by adjusting intracellular osmotic pressure (Claussen, 2005). Accumulation of compatible osmolytes such as soluble sugars also helps plants to overcome abiotic stresses by maintaining osmotic turgor (Grover *et al.*, 2014). However, our results showed no significant change in proline and sugar content after inoculation under salt stress. In another way, it can be interpreted that the plant could establish other salt tolerance mechanisms such as the production of antioxidant enzymes, stress hormones, and the overexpression of genes involved in salt stress tolerance *e.g.*, up-regulation of the aquaporin gene family (Gond *et al.*, 2015). This can be explained by our results, which showed increased production of antioxidant enzyme (POD) utilizing an alternative mechanism to resist salt stress. Further, in a similar study by Abdel Latef *et al.* (2021) *A. chroococcum* strain mitigated salt stress in canola plants grown in saline soil but the inoculation did not show any difference in proline and soluble sugar contents; however, there was an increase in POD activity by 121.7%.

PGPR are also known to mediate induced systemic resistance, which is associated with the induction of various defense enzymes like β-1, 3-glucanase, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidase (PO). Accumulation of pathogenesis-related (PR) proteins viz. β-1, 3-glucanase is known to be associated with systemic

acquired resistance (SAR) in plants (Meena *et al.*, 2000). Various studies have shown that PR-proteins are induced in plants upon treatment with PGPR (Adhipathi *et al.*, 2014; Meena *et al.*, 2000; Singh & Jha, 2017). The production of various defense enzymes in the presence of W4ii might illustrate its role in the generation of resistance to pathogen infection. The increased level of β-1, 3-glucanase in the plant roots might play a key role in pathogen suppression in bacterium-primed plants. Again, β-1, 3-glucanase have the potential to hydrolyze β-1, 3-glucanase, which is a major component of the fungal cell wall. Moreover, glucanase release elicitors from the walls of fungi, which, in turn, stimulate various defense responses in plants (Meena *et al.*, 2000). Therefore, plants can be challenged with fungal pathogen to further evaluate the improvement of plant's ISR when treated with *A. chroococcum* W4ii. Our results also corroborate with the research work where *A. chroococcum* treated plants showed higher peroxidase and β-1, 3-glucanase enzyme activities in the cucumber plants (El-Borollosy & Oraby, 2012).

Finally, the isolated strain W4ii showed efficient colonization to the plant root. It indicates that the ability of colonization of the isolate can provide the concentrated benefits to plants as it creates a close relationship with the plant host than rhizosphere and modulates the defense response to cope with hostile conditions. For more prolonged activity the bacterium needs to have better survival, which is achieved by effective colonization that provides protective environment (Hardoim *et al.*, 2008).

Apart from raising nutrient availability to plants and various PGPR properties, *A. chroococcum* W4ii produces cell-associated black pigment known as catechol melanin, which is thought to be a protective mechanism for aeroadaptation in Na^+ dependent strains/minimizing oxidative stress (Shivprasad & Page, 1989). Many research studies have concluded that the bacterium provides salinity tolerance to various plant species especially in wheat (Alkhalifah *et al.*, 2018; Allam *et al.*, 2018; Chaudhary *et al.*, 2013; Silini *et al.*, 2016), which is in accordance with our present study. In addition to that, the strain has also been used in consortium with other PGPR for further improvement in plant growth and stress resistance (Alkhalifah *et al.*, 2018; Kumar Bhuyan *et al.*, 2015; Yousefi *et al.*, 2017).

Conclusions

Technosoils are valuable sources of salt tolerant PGPR. In the present study, *A. chroococcum* W4ii strain isolated from the technosoils significantly improved the biochemical parameters of winter wheat plants and thus allowed them to cope with imposed salinity stress. The bacterium can very well be used for growing crops in the reclaimed land with salinity problems. Use of this PGPR may be ideal for reducing chemical fertilizer in the agricultural fields to reduce its impact on environmental health. Source of the isolation also makes this strain more robust as it can survive extreme environments and its reproducibility of results to mitigate salt stress makes it a perfect tool for increasing wheat yield under salinity conditions. Future research on this topic could explore the following areas: i) conducting field trials to validate the effectiveness of *A. chroococcum* W4ii in real-world agricultural settings with varying levels of salinity; ii) scaling up the application of this PGPR to larger agricultural areas to assess its practicality and feasibility, iii) conducting comparative studies to test the effectiveness of *A. chroococcum* W4ii with other salt-tolerant PGPR strains or agricultural practices aimed at mitigating salinity stress. This would provide a broader perspective on the best approaches to enhance crop yields under saline conditions.

Ethics and consent

Ethical approval and consent were not required.

Data availability

Underlying data

NCBI GenBank: *Azotobacter chroococcum* strain W4ii 16S ribosomal RNA gene, partial sequence. Accession number OL348496, https://identifiers.org/ncbi/insdc:OL348496 (Binod Kumar & Kalwasińska, 2021).

RepOD: Application of halotolerant Azotobacter chroococcum W4ii isolated from technosoils to mitigate salt stress in wheat plant. https://doi.org/10.18150/10SRTX (Kalwasińska, 2023).

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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Publisher Full Text

Open Peer Review

Current Peer Review Status: ✔ ✘ ✔

Version 4

Reviewer Report 10 September 2024

https://doi.org/10.21956/openreseurope.20020.r44062

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✔ **Hemant Dasila**
[1] Eternal University, Baru Sahib, Himachal Pradesh, India
[2] G.B. Pant University of Agriculture and Technology, Pantnagar, India

Manuscript is refined in a good manner after revision and fit for indexing from my end.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Agriculture Microbiology, Applied Microbiology, Biocontrol, Bioremediation, Microbial Biotechnology and Abiotic stress.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 3

Reviewer Report 14 August 2024

https://doi.org/10.21956/openreseurope.19283.r42677

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✔ **Hemant Dasila**
[1] Eternal University, Baru Sahib, Himachal Pradesh, India
[2] G.B. Pant University of Agriculture and Technology, Pantnagar, India

Authors have tried to address the issue of high salinity in the region which is affecting plant

growth and promotion. Overall manuscript is fit for indexing with some small queries from my end and some minor changes as follows;
1) Abstract is kind of general it should be precise according to the results and these results should be summarized in abstract.
2) Authors have used the term hypothesis which may be null or alternative hypothesis. Did Authors have applied any statistical analysis to test the hypothesis.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Agriculture Microbiology, Applied Microbiology, Biocontrol, Bioremediation, Microbial Biotechnology and Abiotic stress.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Sep 2024

Agnieszka Kalwasińska

Dear Professor Hemant Dasila, Thank you for taking the time to review our work and for providing valuable feedback. Your comments have been helpful in improving our manuscript. Please find our responses to your comments below:
1) Abstract is kind of general it should be precise according to the results and these results should be summarized in abstract.
Answer: The results have been summarized and highlighted in the improved abstract part as per your suggestion.
2) Authors have used the term hypothesis which may be null or alternative hypothesis. Did Authors have applied any statistical analysis to test the hypothesis.
Answer: Yes, the statistical analysis was performed for the experiments conducted in the

present work and described under the title "**Statistical analysis**" in Methods section. The difference between each treatment and control group was analyzed by unpaired t-test or Mann-Whitney test using PAST statistical software package (version 4.03) (RRID:SCR_019129). The difference was considered significant if $P \leq 0.05$. Best regards, Agnieszka Kalwasińska

Competing Interests: No competing interests were disclosed.

Version 2

Reviewer Report 20 May 2024

https://doi.org/10.21956/openreseurope.17765.r37347

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Kailash Chand Kumawat
Department of Industrial Microbiology, Jacob Institute of Biotechnology and Bioengineering, Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad, Uttar Pradesh, India

Title: Title should be reframed and shortened according to study.
Abstract: Abstract needs to be reframed. Also, add field's trial experiment with multifarious PGP traits (at least two years trials based study) and compare under normal soil a minimum of two years field study will be required and then conclude your study because field environmental condition are totally different from pot experiment.
Introduction: Introduction part is very simple and descriptive, so that improve your introduction part with some mechanistic part and justify your study properly with outcomes.
Material and methods: Material and methods are not up to date, please add your two years field study and compare in the normal soil conditions with various plant growth attributes, symbiotic efficiency.
Results: Results are not up to date. Results should be reframe and try to short with important results in the text and remaining mention in the tables and figures. Check all the tables and figures in the text carefully.
Discussion: Discussion part is very poor, improve discussion part and support your study with mechanistic part. Add only recent study in the discussion part to support study with mechanistic part.
Conclusion: Conclusion should be modified after incorporation of all the suggestions, field study results for the farmer communities.
References: References should be reframed according to journal guidelines and check carefully all references in the text as well as references list.
Overall recommendations: According to my opinion this study not completed without two years field study and compared with normal and saline soil conditions under field condition because

field environmental condition are totally different from pot study.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and does the work have academic merit?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant Microbial Interactions

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 22 May 2024

Agnieszka Kalwasińska

Dear Professor Kailash Chand Kumawat,

Thank you very much for your valuable comments on our manuscript. Here, we present our responses to your comments.

Title: Title should be reframed and shortened according to study. Using halotolerant *Azotobacter chroococcum* W4ii from technosoils to mitigate wheat salt stress
Ans: The title has been reframed and shortened accordingly.

Abstract: Abstract needs to be reframed. Also, add field's trial experiment with multifarious PGP traits (at least two years trials based study) and compare under normal soil a minimum of two years field study will be required and then conclude your study because field environmental condition are totally different from pot experiment.
Ans: Thank you for the suggestion. However, the project related to this study has concluded, so the suggested study cannot be continued. More importantly, this study was

part of a larger research project where a range of PGPR were isolated from the technosoil. Other novel strains were also selected as potential bio-stimulants and subsequently chosen for trial-based studies under field conditions. *Azotobacter chroococcum* is an established PGPR; therefore, we have only reported its presence in the technosoil as a potential PGPR. It can be further studied for a field trial.

Introduction: Introduction part is very simple and descriptive, so that improve your introduction part with some mechanistic part and justify your study properly with outcomes.
Ans: Thank you for the suggestion. However, our present study was only a part of the project and indeed quite simple. We did not use any mechanistic approach, and the introduction section aptly describes the idea behind this study..

Material and methods: Material and methods are not up to date, please add your two years field study and compare in the normal soil conditions with various plant growth attributes, symbiotic efficiency.
Ans: As stated above.

Results: Results are not up to date. Results should be reframe and try to short with important results in the text and remaining mention in the tables and figures. Check all the tables and figures in the text carefully.
Ans: All the tables and figures in the text have been carefully checked as per your suggestion. Only the important results are mentioned in the text.

Discussion: Discussion part is very poor, improve discussion part and support your study with mechanistic part. Add only recent study in the discussion part to support study with mechanistic part.
Ans: As stated above, the present study cannot be continued, and no mechanistic research has been conducted in this study.

Conclusion: Conclusion should be modified after incorporation of all the suggestions, field study results for the farmer communities.
Ans: As stated above, the present study cannot be continued further as it was only a part of a project that is already completed.

References: References should be reframed according to journal guidelines and check carefully all references in the text as well as references list.
Ans: The references have been checked as per your suggestion and are in accordance with the journal guidelines.

Overall recommendations: According to my opinion this study not completed without two years field study and compared with normal and saline soil conditions under field condition because field environmental condition are totally different from pot study.
Ans: We completely agree with your opinion regarding our present manuscript. However, A. chroococcum has already been extensively studied as a PGPR. Therefore, we limited our study to pot experiments. In our conclusion, we mentioned that further studies could be conducted in real-world agricultural settings with varying levels of salinity and other scopes.

Separately, we have conducted field studies with other novel strains isolated from the same technosoils. In the future, we can propose another project to continue our studies based on your suggestions. We thank you for your valuable comments.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 August 2023

https://doi.org/10.21956/openreseurope.17765.r34425

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Megha Kaviraj
University of Birmingham, Birmingham, England, UK

Yes, the authors' significant modifications and revisions can be now approved for scientific standard publication.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biological nitrogen fixation, plant-microbe interaction, soil microbiology, Biofertilizer, Nitrogen cycle, DNRA, Molecular Biology, Metagenomics, Bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 July 2023

https://doi.org/10.21956/openreseurope.17091.r33560

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?

Megha Kaviraj
University of Birmingham, Birmingham, England, UK

1. The article is quite an interesting and details study. However, the abstract should be more focused on a line of research gap, hypothesis and a few best quantitative percentages of improvement data.

2. The authors should specify in the methods section at what point or how many days after wheat growth a soil sample was taken. Additionally, the fertilizer regime for that scenario should be included.

3. The data that was not presented in the bar graph can be incorporated in tabular form for better understanding.

4. The organism's name should be italics, please rectify it throughout the manuscript including graphs.

5. "The bacterial cell mass was collected and mixed with 10% skimmed milk. The bacterial cell mixture with skimmed milk was then lyophilized to get the powered formulation to be applied in soil". What is the reason behind using carrier-based solid formulation instead of liquid formulation? How economical and viable to make it solid with skimmed milk?

6. The last line of future study in the conclusion section is very generalized please make it robust.

7. Please check spelling and English corrections for better readability.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biological nitrogen fixation, plant-microbe interaction, soil microbiology, Biofertilizer, Nitrogen cycle, DNRA, Molecular Biology, Metagenomics, Bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Jul 2023

Agnieszka Kalwasińska

Dear Professor Megha Kaviraj, Thank you for your support in reviewing our manuscript. We greatly appreciate your insightful comments. We are committed to incorporating your feedback to improve the manuscript, and we aim to meet your expectations with the revised version. Sincerely, Agnieszka Kalwasińska

Answers for the Revewer's comments

Answer 1: The Abstract has been changed accordingly

Answer 2: The information regarding the soil used for the study along with its physicochemical properties has been added to the Materials and Methods section.

Answer 3: All data presented in the manuscript are in figures (Fig.1 – Fig.12), additionally, the details can also be inspected in Excel files in the repository RepOD https://doi.org/10.18150/10SRTX (Kalwasińska, 2023).

Answer 4: The organism's name was checked throughout the manuscript and all figures were improved accordingly.

Answer 5: The application of the carrier-based solid bacterial formulation with skimmed milk instead of a liquid formulation has been explained in the Materials and Methods section.

Answer 6: The future research has been adjusted accordingly.

Answer 7: The entire manuscript has been checked for spelling, and corrections were made.

Competing Interests: No competing interests were disclosed.