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Unlocking the biofortification potential of *Serratia marcescens* for enhanced zinc and iron content in wheat grains

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ABSTRACT: In light of current challenges, there's an urgent call for innovative solutions to not only ensure food security but also tackle the pervasive issue of malnutrition amidst mounting environmental pressures. Biofortification, the process of enhancing the nutrient content of crops, is a critical strategy to address malnutrition worldwide. Within this framework, Plant Growth Promoting Rhizobacteria (PGPR) play a vital role in biofortification by improving nutrient uptake, solubilizing insoluble nutrients, and promoting plant growth, thereby contributing to the development of nutrient-rich crops. In this investigation, bacteria were isolated from rhizospheric soil and screened for their potential to solubilize zinc (Zn). Among the isolated rhizobacterial strains, one demonstrated high Zn solubilization potential (48.33µg/mL) during quantitative estimation was selected for further study and identified as *Serratia marcescens* SCHN1 through molecular identification. Furthermore, selected isolate *S. marcescens* SCHN1 was found to be positive for multiple PGPR traits including phosphate solubilization, indole-3-acetic acid (IAA), siderophore and ammonia (NH_3) production. Subsequently, a pot trial was conducted with wheat seeds inoculated with the selected PGPR strain. Results showed that wheat plants inoculated with the *S. marcescens* SCHN1 exhibited significant increase in plant height by 11.99% over control. Moreover, the micronutrient content, including zinc (80 mg/kg) and iron (593.5 mg/kg) in wheat grains were significantly improved upon treatment over control group. These findings underscore the importance of PGPR in biofortification efforts aimed at enhancing crop nutrient content and overall plant growth, thus offering promising avenues for addressing global food security challenges.

Key words: Biofortification, malnutrition, micronutrient, PGPR, zinc solubilization
Abbreviations: Ammonia= NH_3; Indole acetic acid= IAA; Iron= Fe; PGPR= Plant Growth Promoting Rhizobacteria; Zinc= Zn

Zinc (Zn) stands as a pivotal element crucial for the well-being of biological system including, plant, human and microorganisms for supporting vital biological processes and contributing to overall health and development (Upadhayay *et al.*, 2022a). It has a direct or indirect influence on various aspects of plant physiology, such as growth, maturity, vigor, and yield. Zn is intricately involved in key plant functions, including auxin synthesis and the catalysis of the photochemical reaction of chlorophyll. Moreover, it bolsters the stability of biological membranes and is crucial for the activity of various enzymes. In context to the human and organism health, Zn is required in trace amounts to support proper physiological functions; recognized as a vital mineral for overall well-being (Kumar *et al.*, 2019). However, the availability of Zn in soils is diminishing due to factors like low organic matter, excessive fertilization, inadequate recycling of crop residues, cultivation of high-yielding crop varieties, and intensive cropping patterns (Upadhayay *et al.*, 2022b). The scarcity of Zn in soil presents a significant challenge, resulting in deficient crop yields due to its crucial role in various plant metabolic processes. Zn deficiency disrupts essential functions like photosynthesis and nitrogen metabolism in plants, leading to impaired flowering, stunted fruit development, and reduced carbohydrate synthesis, thereby delaying crop maturity and decreasing both yield and grain nutritional quality. Recent studies highlight a concerning global trend, with approximately half of the world's population facing Zn deficiency, exacerbated by the prevalence of low available Zn in soils used for cereal production, further contributing to diminished grain yield and nutritional value (Mumtaz *et al.*, 2017). Addressing the issue of Zn deficiency, there is a rising focus on micronutrient biofortification of staple grain crops in developing nations, aiming to enhance nutritional quality and combat widespread deficiencies. Beneficial free-living soil bacteria, specifically plant growth-promoting rhizobacteria (PGPR), have shown promise in improving plant health and bolstering yield (Singh *et al.*, 2017; Khan *et al.*, 2022a). PGPR fulfil multifaceted roles in sustainable agriculture as they reside within the rhizosphere, encompassing root surfaces and establishing symbiotic relationship with plant roots to enhance overall plant growth and health (Singh *et al.*, 2013; Shaikh and Saraf, 2017; Bundela *et al.*, 2023). The solubilization of metal salts constitutes a significant trait of PGPR, facilitating the mobilization of compounds accessible to plants. Various PGPR, including strains from genera such as *Serratia*, *Bacillus*, *Pseudomonas*, and have been identified as effective Zn solubilizers, augmenting plant growth by colonizing the rhizosphere and converting complex Zn compounds into simpler forms accessible to plants (Yadav *et al.*, 2020; Singh *et al.*, 2022; Upadhayay *et al.*, 2022a). The utilization of PGPR presents a promising and environmentally-friendly approach, serving as a viable substitute for chemical fertilizers, pesticides, and supplements, contributing to sustainable agricultural practices and promoting soil health.

The crop, wheat (*Triticum aestivum* L.) is recognized as one of the world's foremost staple crops, second only to maize in global production. Soil nutrients, encompassing primary, secondary elements and micronutrients constitute significant factors limiting wheat production's yield potential (Pandey *et al.*, 2020). Nonetheless, low levels of micronutrients such as iron (Fe) and Zn in wheat grain can engender micronutrient deficiencies in individuals whose diets mostly consist of cereals like wheat (Ali and Borrill, 2020). Introducing these strains into crops can establish non-traditional pathways for producing nutritious food (Upadhayay *et al.*, 2019). Inoculating crops with Zn-solubilizing bacteria (ZSB) not just addresses malnutrition by elevating nutrient concentrations in grain part but also serves as an alternative to Zn fertilizers in soils with high amount of inaccessible Zn content (Upadhayay *et al.*, 2024). The aim of the present study is to explore the potential of Zn-solubilizing microbes in enhancing micronutrient content in crops. By investigating the efficacy of these microbes, our effort is to formulate sustainable agricultural practices capable of addressing micronutrient deficiencies in crops, thereby improving nutritional quality. This research endeavours to offer invaluable insights into harnessing the beneficial impact of Zn-solubilizing microbes for enhancing overall crop health and nutritional value.

MATERIALS AND METHODS

The bacterial culture employed in this investigation were obtained from the rhizospheric soil of hilly regions in Uttarakhand. Ten isolates were initially screened for their Zn solubilization capabilities, leading to the identification of the most promising isolate, SCHN1, was chosen for subsequent experiments. Utilizing 16S rRNA sequencing, the selected isolate was identified as *Serratia marcescens* and was subsequently archived in the gene bank with the accession number OR473077. Further characterization of this strain included a study of its PGPR attributes, followed by a pot experiment conducted on the staple crop wheat (*Triticum aestivum*) at Biofortification Lab, Department of Microbiology, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar.

Zinc solubilizing potential

To evaluate the Zn solubilization capability, the chosen bacterial isolates was spot-inoculated on the solid basal medium as outlined by Saravanan *et al.* (2004). The medium composition included dextrose (10.0 g), $(NH_4)_2SO_4$ (1.0 g), KCl (0.2 g), K_2HPO_4 (0.1 g), $MgSO_4$ (0.2 g), Agar (15.0 g) with a pH of 7.0 supplemented with insoluble Zn compounds (zinc oxide (ZnO) and zinc carbonate ($ZnCO_3$) at 0.1%;), autoclaved at temperature and pressure of 121^0C and 15 psi respectively. Clearing zone diameters around the bacterial colonies were measured every three days for a duration of up to fourteen days (Suyal *et al.*, 2023).

For quantification, 1 mL of actively grown bacterial culture with a cell load of 10^8 cells mL^{-1} was inoculated into a liquid basal medium (50 mL) supplemented with ZnO (0.1%). After incubation, samples were taken at 0, 7, and 15-day intervals, centrifuged to eliminate cell debris, and then 10 mL of the culture supernatant was filtered. The available Zn content was determined using an atomic absorption spectrophotometer (AAS) (Suyal *et al.*, 2023). Simultaneously, the effect of solubilization on the medium's pH during the bacterial isolate's growth was also assessed.

Plant growth promoting attribute assessment

The selected bacterial strain was evaluated for its ability to promote plant growth through solubilization of phosphate, siderophore and ammonia production and indole acetic acid synthesis. For the qualitative assessment of phosphate solubilization, Pikovskaya medium containing tricalcium phosphate was utilized, and the diameter of the halo zone around the colony growth were observed over a 5-day period. The level of phosphate solubilization was quantitatively assessed using Pikovskaya broth medium, and the available phosphate content was measured according to the methodology detailed by Ryan *et al.* (2001). Production of low molecular weight compound, siderophore was evaluated qualitatively on Chrome Azurol (CAS) agar medium, where the presence of a distinct yellow-orange halo zone surrounding the bacterial growth indicated positive results, while quantitative analysis was performed using the Chrome Azurol S (CAS) shuttle assay (Saraf *et al.*, 2013). Additionally, the synthesis of indole acetic acid by the chosen bacterial isolate was assessed via the Salkowski reagent method, with the intensity of the pink colour at 530 nm measured spectrophotometrically, following calibration using a standard IAA stock solution (100 mg/mL) (Bric *et al.*, 1991). Ammonia (NH_3) production was detected by the addition of one millilitre of Nessler's reagent to a 72-hour old bacterial culture grown in peptone broth (10 mL), where positive samples exhibited a distinct yellowish-brown colouration, indicating the presence of ammonia (Cappuccino and Sherman, 1992).

Seed bacterization and pot trial experiment

Seeds of a cultivable wheat variety (UP 2865) underwent surface sterilization using 0.1% mercuric chloride solution for 3 min followed by 70% ethanol for 3 min and were then rinsed eight times with sterile distilled water as described by Singh *et al.* (2010). Subsequently, the seeds were treated with overnight grown bacterial inoculum having optical density (10^5 to 10^6 Colony Forming Unit) along with 0.5% carboxymethylcellulose (CMC) to provide adhesiveness. The pot experiment conducted in a 5kg pots filled with 4kg soil with three replications for each treatment. Bacterized seeds were sown at the depth of 2.5cm in the soil with the help of sterilized forceps. To provide moisture, plants were watered at alternate days. Agronomical parameters such as plant height of uninoculated (control) and inoculated plants were measured at the time of harvest.

Analysis of Zn and Fe concentration in harvested wheat grains

The harvested wheat grains underwent further analysis to determine their available Zn and Fe content. In this process, a representative grains sample (100 mg) from both the treatment and control groups was finely ground into an amorphous powder using a mortar and pestle. The powdered samples were then subjected to digestion on a hotplate in a tri-acid mixture consisting of nitric acid (10 parts), perchloric acid (4 parts), and sulfuric acid (1 part) in a volume-to-volume ratio. Afterward, 6N hydrochloric acid (5 mL) was added to the mixture. The process of digestion continued until the entire plant material turned colourless. Subsequently, the volume was makeup to 50 mL with distilled water. The resulting extract was then filtered through Whatman no. 42 filter paper and finally analysed for Fe and Zn content using an atomic absorption spectrophotometer, as outlined by Estefan *et al.* (2013).

Statistical analysis

Based on the experimental design, the data from the plant study were meticulously analysed in triplicate and subjected to Analysis of Variance (ANOVA). Statistical analysis was executed utilizing the SPSS statistical package to accurately quantify and evaluate the sources of variation. Subsequently, treatment means were compared at a significance level of 5% to ascertain the presence of any significant differences.

RESULTS AND DISCUSSION

Zinc solubilizing potential of selected bacterial strain

In the present study selected plant growth promoting rhizobacteria (PGPR) is isolated and evaluated for Zn solubilization potential. During the plate assay, it demonstrated a significant solubilization of insoluble Zn compounds (ZnO and $ZnCO_3$) and the bacterium was assigned the code SCHN1. Results from *in vitro* studies revealed that the selected strains solubilize ZnO and $ZnCO_3$ and developed a halo zone with diameter of 5.47±0.21 and 3.03±0.13 cm, respectively (Figure 1 & 2). Furthermore, its robust ability of efficient ZnO solubilization was further confirmed through quantitative estimation. The findings of the quantitative estimation demonstrated that the tested rhizobacterial strain dissolved 48.33±2.47µg/mL of ZnO in broth medium (Table 1). Furthermore, the introduction of Zn-solubilizing bacterial isolates resulted in a notable reduction in the pH of the broth medium comparison to the control, indicating their potential role in altering the chemical environment (Table 1).

Table 1: Potential of rhizobacterial strain SCHN1 to solubilize ZnO in liquid medium

Bacterial isolate	Quantitative Zn solubilization (µg/mL)	Broth pH
SCHN1	48.33±2.47	3.9±0.23

Note: µg/mL = microgram/milliliter; The values represented in the table are expressed as means of three replicates ± standard deviation (SD).

Plant growth promoting attributes

Plant growth promoting characteristics including, phosphate solubilization, production of siderophore, indole acetic acid, and ammonia results are depicted in Table 2. The bacterial isolate exhibited the ability to solubilize phosphate, as evidenced by the zone formation in the agar medium supplemented with insoluble source, tricalcium phosphate. Furthermore, quantitative estimation revealed that bacteria could solubilize 116.96±3.43µg/mL phosphate in a liquid medium. Similarly, the tested bacteria exhibited positive results for qualitative siderophore production, and produce siderophore unit 32.01±2.48 in liquid medium during quantitative estimation. While, the production of IAA was accounted in range of 10.34±0.13µg/mL of the test isolate. Moreover, SCHN1 was also found positive for ammonia production.

Effect of bacterial strain on wheat plant height

During time of harvest plant height was calculated to observed the influence of test isolates on the plant growth and development. Results demonstrated that introduction of Zn solubilizing isolates notable increased plant height in comparison to the uninoculated control. SCHN1 treated plants showed height of 82.87±3.96cm which was significantly higher than control plants (74±2.98cm) (Figure 3).

Micronutrient concentration in wheat grains

In this study, the primary objective was to isolate and characterize Zn-solubilizing strains to explore their potential to enhance the biofortification of Zn and Fe in wheat under a pot trial experiment, which demonstrated that the inoculation of these strains not only enhanced plant growth but also influenced the levels of Zn and Fe in the plants (Figure 4). The findings from SCHN1 treated plants revealed that the concentration of Fe and Zn were found to be 593.5 and 80.00 mg/kg respectively. It was

Table 2: Plant growth promotory potential of rhizobacterial strain SCHN1

Bacterial isolate	Qualitative phosphate solubilization	Quantitative phosphate solubilization (µg/mL)	Qualitative siderophore production	Quantitative siderophore unit	IAA production (µg/mL)	NH_3 production
SCHN1	+	116.96±3.43	+	32.01±2.48	10.34±0.13	+

Note: µg/mL = microgram/milliliter; The values represented in the table are expressed as means of three replicates ± standard deviation (SD).

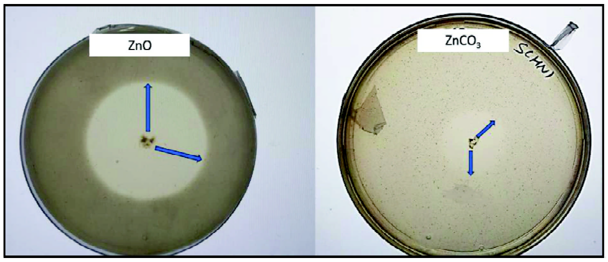

Fig. 1: Formation of halo zone by rhizobacterial strain SCHN1 to solubilize ZnO and $ZnCO_3$ on solid agar medium

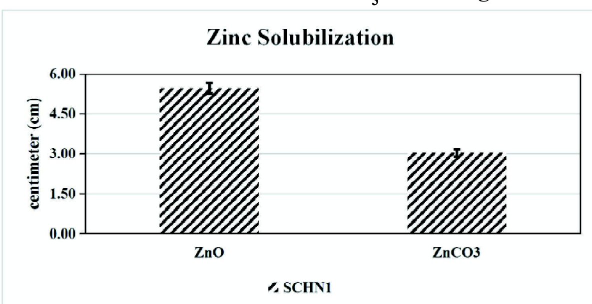

Fig. 2: Potential of rhizobacterial strain SCHN1 to solubilize different zinc source on solid agar medium

significantly higher than control which accounts 444.50 and 56.50 mg/kg for Fe and Zn respectively.

DISCUSSION

The utilization of plant growth-promoting rhizobacteria has become increasingly recognized for their instrumental role in fostering plant growth and bolstering biofortification endeavors, particularly in staple food crops like wheat, maize, rice, pearl millet, and various other agricultural produce (Khan *et al.*, 2022a). These beneficial bacteria colonize the plant rhizosphere, forming symbiotic relationships with plants and exerting various mechanisms to stimulate growth. By producing phytohormones, such as auxins and cytokinin, PGPR can enhance root development and nutrient uptake, thus fostering improved plant vigor (Singh *et al.*, 2018). Additionally, some PGPR strains possess the ability to solubilize nutrients such as phosphorus, Fe, and Zn in the soil, making them more accessible to plants (Prasad *et al.*, 2019; Serawat *et al.*, 2022). This enhanced nutrient availability not only promotes plant growth but also contributes to biofortification efforts by increasing the accumulation of essential minerals in edible plant

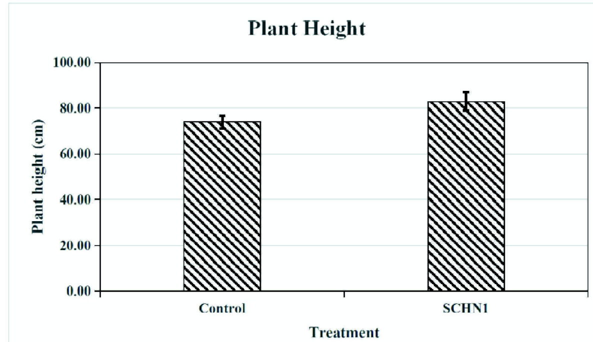

Fig. 3: Effect of bacterial inoculation on plant height of wheat (*Triticum aestivum*)

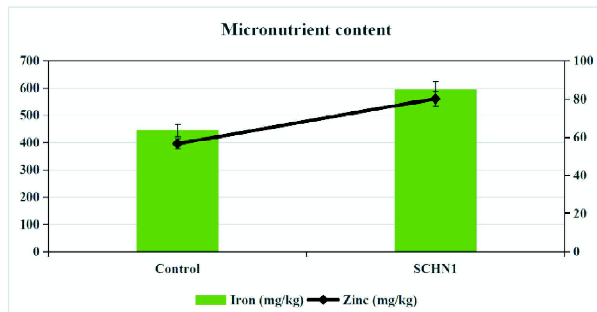

Fig. 4: Effect of bacterial inoculation on available Zn and Fe in wheat grains (*Triticum aestivum*)

parts (Upadhayay *et al.*, 2022c). Harnessing the potential of PGPR offers a sustainable and eco-friendly approach to enhance agricultural productivity while simultaneously addressing malnutrition through biofortification strategies (Khan *et al.*, 2022b). Our study therefore focused on the utilization of Zn solubilizing rhizobacterial strain to enhance plant growth and development and increase micronutrient content in wheat grains. However, the tested bacterial strain also showed other plant growth-promoting (PGP) traits including, phosphate solubilization, production of siderophore, IAA, and ammonia. Various mechanisms contribute to Zn solubilization by PGPR, including the excretion of metabolites like organic acids, proton extrusion, and the production of chelating agents. Additionally, PGPR are capable of producing inorganic acids such as, sulfuric acid, nitric acid, and carbonic acid, contributing to the solubilization of metals and further enhancing nutrient availability for plant uptake (Khan *et al.*, 2019). The efficacy of Zn solubilization by tested strains is evident from the data, highlighting their efficient solubilization potential. Similar results were obtained in a study performed by Upadhayay *et al.* (2022a), where *Serratia* sp. FMAR105 exhibited superior Zn solubilization, and forming halo zone of different sizes. The observed formation of halo zones with varying sizes, namely 1.63cm, 1.50cm, and 1.2cm on mineral salt media containing ZnO, $ZnCO_3$ and $ZnPO_4$ suggests Zn solubilization potential facilitated by bacterial secretion of organic acids (Upadhayay *et al.*, 2022a). Additionally, mechanisms such as proton secretion and siderophore production, as documented in numerous studies, may contribute to the overall enhancement of Zn bioavailability (Saravanan *et al.*, 2011; Kamran *et al.*, 2017; Singh and Prasanna, 2020; Suyal *et al.*, 2023). The tested Zn solubilizing rhizobacterial strain was also found positive for phosphate solubilization, siderophore production, indole acetic acid production, and ammonia production. Phosphorus (P) stands out as a crucial nutrient essential for optimal plant growth (Singh *et al.*, 2011). Microorganisms in the rhizosphere exhibit a remarkable ability to increase the accessibility of inorganic phosphorus by releasing protons, organic acids, and specialized ligands, thereby facilitating the mobilization of phytate, an organic form of phosphorus, via the production of phytase enzymes (Granada *et al.*, 2018). In our current investigation, bacterial isolate *S. marcescens* SCHN1 demonstrated efficient phosphate solubilization in both solid and liquid mediums. Similar outcomes were noted by Gupta *et al.* (2022), who conducted qualitative and quantitative assessments of bacterial isolates, observing the formation of halo zones surrounding the bacterial colonies on Petri plates due to organic acid production, identifying them as proficient phosphate solubilizers. Various bacterial strains produce siderophore compounds in response to Fe deficiency, crucial for their competitive fitness in colonizing plant roots and competing for Fe within the rhizosphere (Kumar *et al.*, 2014). In our study, qualitative and quantitative assessments confirmed the bacterial isolate's positive siderophore production. Comparable findings were reported by Shaikh and Saraf (2017), where *Exiguobacterium aurantiacum* strain MSZT10 exhibited substantial zone formation in agar medium and efficient solubilization in liquid medium. Additionally, our bacterial isolate demonstrated positive results for IAA and NH_3 production. The IAA production capability aligns with previous studies (Abaid-Ullah *et al.*, 2015; Defez *et al.*, 2019), known for promoting primary root elongation and the development of lateral/adventitious roots. Another noteworthy trait of PGPR is ammonia (NH_3) production, indirectly influencing plant growth. Furthermore, the study evaluated the selected Zn-solubilizing rhizobacterial strain's potential for wheat growth promotion under pot trial conditions. Inoculation results indicated that strain SCHN1 significantly increased wheat plant height compared to uninoculated controls. This growth enhancement could be attributed to the ability of rhizobacterial isolates to enhance nutrient availability through P and Zn solubilization, siderophore and phytohormone production, as well as antagonistic activity against plant pathogens. Our findings are consistent with previous studies (Gandhi and Muralidharan, 2016; Khatoon *et al.*, 2020). Auxin production (IAA) by rhizobacterial isolates is recognized for improving root growth, consequently augmenting the absorptive surface area of roots for improved nutrient uptake (Mumtaz *et al.*, 2017). Malnutrition characterized by deficiencies or imbalances in a person's intake of nutrients poses a significant global challenge, affecting population worldwide (Bouis and Saltzman, 2017). Micronutrients like Zn and Fe play crucial roles in maintaining human health, supporting various physiological functions such as immune system regulation, cognitive development, and energy metabolism. However, deficiencies in these micronutrients are prevalent, particularly in developing countries, leading to serious health consequences such as impaired growth and cognitive development, compromised immune function, and increased susceptibility to diseases (Nieder *et al.*, 2018). PGPR offers a promising solution to address this issue. PGPR are beneficial bacteria that colonize plant roots and enhance plant growth and health through various mechanisms, including improving nutrient uptake and bioavailability. By promoting plant growth and increasing the nutrient content of crops, PGPR can contribute to combating Zn and Fe deficiencies in populations reliant on plant-based diets, ultimately improving public health outcomes (Dhuldhaj and Pandya, 2017). In our study, the selected PGPR improved the micronutrient content (Zn and Fe) in the wheat grains in comparison to the uninoculated plants. Similar studies were reported by other researchers in which Zn solubilizing strains belongs to the genera, *Bacillus* and *Paenarthrobacter* were found to be promising for enhancing micronutrient content particularly Zn in wheat grains (Yadav *et al.*, 2022). Moreover, Khan *et al.* (2022a) reported in their study that rhizobacterial strain PWR 28 inoculation significantly improved Fe content in wheat grains i.e., 26.72±1.52 mg/kg over control (20.22±1.79 mg/kg). Thus, our study demonstrates that Zn solubilizing rhizobacterial strain, *S. marcescens* SCHN1 showed its efficacy in wheat cultivation by efficiently exhibiting PGPR traits such as Zn and phosphate solubilization, production of siderophore, NH_3 and IAA. Through this plant-microbe relationship, SCHN1 not only boosts plant growth but also enhances the uptake of essential micronutrients, ultimately contributing to healthier and more resilient wheat crop.

CONCLUSION

The current research underscores the significant potential of the *Serratia marcescens* SCHN1 isolate in promoting wheat growth by effectively dissolving Zn from insoluble sources. Its multiple traits conducive to plant growth promotion and efficient root colonization highlight its promising role as an inoculant for enhancing wheat grain productivity and nutrient quality. The findings suggest that further evaluation of these multi-trait strains could pave the way for their utilization as bio-inoculants to combat Zn deficiency in plants, consequently addressing Zn malnutrition in humans. This study opens avenues for innovative strategies to mitigate malnutrition and improve agricultural sustainability through the application of beneficial microbial agents.

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