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Improved tomato (*Solanum lycopersicum* L.) growth and reduction of fungal pathogens utilising the plant growth-promoting and antifungal *Bacillus albus* NJ01 as a bioinoculant

Nilina James and Mridul Umesh*

Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, 560029 Karnataka, India.
**E-mail: mridul.umesh@christuniversity.in*

Abstract

Rhizobacteria that promote plant growth are crucial for improving the health, growth, and yield of plants. In this study, 14 isolates were obtained and the significance of *Bacillus albus* NJ01 as PGPR for the improvement of growth in tomato (*Solanum lycopersicum*) was assessed, as it showed plant growth-promoting traits like IAA, siderophores and ammonia production, phosphate and zinc solubilization, etc. Its role in increasing crop root and shoot length while avoiding the use of chemical pesticides and fertilizers was also studied. The root length of tomato control plants and plants treated with bioinoculant was found to be 5.58 ± 0.15 and 7.98 ± 0.24 cm, respectively. The shoot length of control plants and plants treated with bioinoculant was found to be 8.25 ± 0.82 and 10.24 ± 0.11 cm, respectively, therefore confirming the potentiality of *Bacillus albus* NJ01 bioinoculant as an able PGPR for improving the growth of tomato.

Key words: Antifungal, *Bacillus albus* NJ01, bioinoculant, plant growth promoting rhizobacteria, *Solanum lycopersicum*, 16s rRNA sequencing

Introduction

Plant Growth Promoting Rhizobacteria (PGPR) are emerging as a sustainable alternative to chemical fertilisers due to their ability to enhance plant growth by significantly improving soil fertility (Shukla and Shaikh, 2023). This improvement is achieved through the modification of soil structure, the augmentation of organic matter content, the enrichment of soil microbiota, and the facilitation of nutrient cycling processes (Bhat *et al.*, 2023). These bacteria secrete a range of metabolites into the rhizosphere, the region of soil that is in close contact with root secretions, that stimulate plant development. The robustness and adaptability of *Bacillus* spp. to diverse soil environments is remarkable.

Bacillus spp. are known to boost plant growth through the production of phytohormones, including indole-3-acetic acid (IAA), cytokinins, and gibberellins. IAA, a crucial auxin, is synthesised by PGPR via two major pathways: the tryptophan-dependent pathway, where tryptophan acquired from the soil is synthesised using the enzyme tryptophanase to produce IAA, and the Indole-3-Acetamide (IAM) pathway, in which indole-3-acetamide is converted to IAA. Cytokinins are produced by the activity of the enzyme isopentenyl transferase (IPT), which catalyses the reaction between dimethylallyl diphosphate and ATP (Ratnaningsih *et al.*, 2023). Further, some *Bacillus* spp., especially *B. subtilis*, can fix nitrogen and increase the availability of this element in nitrogen-deficient soils. This nitrogen fixation is facilitated by rhizosphere colonisation and the expression of an enzyme known as nitrogenase, which is involved in the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3), a form easily assimilated by plants. In addition, PGPR belonging to the *Bacillus* genus have been found to promote plant growth via the solubilisation of vital nutrients, including phosphate, zinc, and potassium. Phosphate and zinc solubilisation occurs through three principal mechanisms: enzymatic production of phosphatase, secretion of siderophores chelated with iron (Fe) and aluminium (Al) to keep phosphate in a soluble form, and acidification through the secretion of organic acids by PGPR. *Bacillus* spp. are also known to be antagonistic towards various plant pathogenic fungi. They produce antibiotics, cell wall-degrading enzymes, volatile organic compounds, induce systemic resistance in plants, and exhibit mycoparasitism, making them effective biocontrol agents. It has been proven that *Bacillus* spp. can activate plant defence mechanisms against pathogens by inducing the production of phytoalexins and other pathogenesis-related proteins through the activation of recognition receptors that trigger immune responses (Randive *et al.*, 2024).

In relation to these benefits, PGPR exhibits antifungal activity, thereby serving as a biological control method against harmful fungal pathogens. This function is crucial in maintaining the health and productivity of plants, especially in agricultural systems where the use of chemical fungicides may have negative impacts on the environment or lead to the development of resistant strains of fungal pathogens (Shukla and Shaikh, 2023). In addition, PGPR also has the ability to tolerate and remediate heavy metal-contaminated soils, such as those contaminated by lead and zinc. They utilise mechanisms that include biosorption, bioaccumulation, and the production of metal-chelating compounds, all serving to reduce the bioavailability of heavy metals and mitigate their toxic effects on plants. Besides, using PGPR is more cost-effective than using chemical fertilisers

because, in most cases, biofertilizers require less frequent application (Goswami *et al.*, 2016).

Tomatoes (*Solanum lycopersicum*) are an important food crop due to their rich content of essential nutrients like vitamins A, C, and E, potassium, and folate. However, tomato cultivation faces several challenges, including diseases and pests, environmental stresses arising from extreme moisture, humidity, temperature, nutrient deficiencies, poor irrigation, water management, soil-borne pathogens and poor pollination. Addressing these challenges at the very early stages of growth is critical for successful cultivation. Besides, it must be noted that the efficiency of PGPR in promoting plant growth depends on the bacterial strain used, the prevailing environmental conditions, and plant species.

This study seeks to address critical gaps in the understanding of PGPR, specifically focusing on *B. albus* NJ01, a relatively underexplored strain. While the beneficial effects of *Bacillus* spp. on plant growth are well-documented, there is limited research on the specific mechanisms through which *B. albus* NJ01 promotes growth in tomato plants. The research questions guiding this study are the specific mechanisms by which *B. albus* NJ01 enhances nutrient availability, phytohormone production, and plant defence in tomatoes.

The effect of *B. albus* NJ01 in providing biocontrol against soil-borne pathogens in tomato cultivation. By investigating these questions, the study aims to fill the existing gaps in the literature and contribute new knowledge on the application of *B. albus* NJ01 as a biofertilizer.

Materials and methods

Collection of the sample: Soil samples necessary for the study were collected from the rhizospheric zone of *Coffea arabica* plants at the Kamath Coffee Estate in Gonikoppal, Kodagu district, Karnataka. These samples were gathered aseptically from the root vicinity after the topsoil was removed (James *et al.*, 2023). The collected soil samples were then transported to the laboratory and stored at 4°C for further analysis.

Isolation of PGPR from soil: Isolation of soil bacteria was done by adding 1 g of collected soil in nutrient broth which was incubated at 37 ℃ overnight for enrichment of PGPR (James *et al.*, 2023). After incubation, the culture was serially diluted up to 10^{-6} dilution and plated on nutrient agar plates. After 24 h of incubation at 37 ℃, different colonies were obtained and preserved after checking the colony morphology.

Screening for plant growth-promoting activity: After isolation, all the morphologically different bacterial colonies obtained from the plate were subjected to different plant growth-promoting assays to screen out the one potential bacterial strain for further studies.

Test for IAA production: Quantitative estimation of IAA was determined using the Salkowski reagent method (James *et al.*, 2023). Overnight culture (1 mL) was added to nutrient broth amended with 1% (w/v) tryptophan. After 24 h of incubation at 37 ℃, the broth contents were centrifuged and the supernatant was collected. 2 drops of orthophosphoric acid (99.9%) (w/v) was added to the tubes after which 2 mL of Salkowski reagent was added. The tubes were incubated in the dark for 30 min and absorbance was measured at 535 nm. Pink colouration in the test tube indicated positive results.

Test for siderophore production: The qualitative assay of Siderophore was determined using the $FeCl_3$ method (James *et al.*, 2023). One millilitre of overnight culture was added to nutrient broth in 1 L of distilled water. After 24 h of incubation at 37 ℃, the broth contents were centrifuged at 10000 rpm for 10 min and the supernatant was collected. Two millilitre of 2 % $FeCl_3$ (w/v) was added to 5 mL of culture. The change of the colour to brown is interpreted as a positive result.

Test for ammonia production: Ammonia production was determined using Nessler's reagent (James *et al.*, 2023). One millilitre of overnight culture was added to peptone water. After 72 h of incubation at 28±2 ℃, the broth contents were centrifuged at 10000 rpm for 10 min and supernatant was collected. two mL of 2 % $FeCl_3$ (w/v) was added to 5 mL of the supernatant. The contents of the test tubes changed to brown colour to indicate positive results.

Test for phosphate solubilisation: The process of assessing phosphate solubilization involved inoculating the isolate on Pikovskaya's agar medium. The plates were placed in a incubator at 28 °C for the next 7 days post-inoculation, after which the zone of clearance was examined (James *et al.*, 2023). The Phosphate solubilisation Index (PSI) was then calculated using the formula: Phosphate Solubilisation Index = Total diameter (colony + halo) / diameter of colony

Test for zinc solubilisation: To assess the isolates' ability to solubilise Zn, Pikovskaya media that had been modified with $ZnCl_2$. After being inoculated with the isolate, the plate was kept at 28 °C for a week. The incubated plates were examined for colonies with a halo around them (Zaheer *et al.*, 2019). The Zinc solubilisation Index (ZSI) was then calculated using the formula: Zinc Solubilisation Index = Total diameter (colony + halo) / diameter of colony

Antifungal assay: The antifungal properties of the isolate were assessed using four fungal plant pathogens: *Aspergillus niger MTCC 4325, Fusarium solani MTCC 2671, Cladosporium cladosporioides MTCC 3483, Talaromyces flavus MTCC 1778*. Fungal discs were prepared by inoculating the fungi onto Sabouraud's Dextrose Agar (SDA), incorporating filter paper discs. The SDA plates were incubated at room temperature for 5 days to allow mycelial growth. Concurrently, separate SDA plates were inoculated with two parallel lines of the bacterial isolate 6 cm apart. These plates were incubated at 37±2°C for 24 hours to facilitate bacterial growth. Following this, the previously prepared fungal discs were placed at the centre of the SDA plates containing the bacterial isolate and incubated at room temperature for 7 days. Control SDA plates, which did not contain the bacterial isolate, were also inoculated with the same fungi. Fungal growth was monitored for all samples, with positive antifungal activity indicated by restricted fungal growth in the presence of the bacterial isolate.

Molecular characterisation of the selected isolate: Out of 14 isolates, the best one that showed positive results for all the plant growth-promoting tests was selected and later 16s rRNA gene sequencing was carried out at Barcode Biosciences, Bangalore, Karnataka, India. The 16S rRNA gene fragment was amplified

using PCR after the genomic DNA was isolated from the culture using agarose gel electrophoresis (1% agarose gel) (Kimura 1980). The sequence was determined using aligner sequence software. With the 'nr' database of the NCBI GenBank database, BLAST was run, and the top 10 sequences were examined based on the maximum identity score. The CLUSTAL ω program was used to align several sequences. With MEGA 10 software, a distance matrix and phylogenetic tree were created (Kumar *et al.*, 2018).

Bioinoculant preparation using *B. albus* NJ01: One hundred millilitres of nutrient broth was used to culture *B.albus* NJ01. By centrifuging the culture at 6000 rpm for 10 min at 4 °C after 24 h, bacteria were removed from the media (Canfora *et al.*, 2022). To eliminate the medium components, the cells were carefully harvested without any cell loss and washed twice in sterile distilled water (SDW). The cleaned cells were resuspended in SDW (100 mL) to achieve a density of 10^4 CFU mL^{-1} rhizobia (Dasgupta *et al.*, 2023).

Treatment of seeds with liquid bioinoculant: The study was conducted using seeds from tomato (Arka-Samrat variety from IIHR, Bangalore, Karnataka, India). After being surface sterilised with 0.1% (w/v) $HgCl_2$, the seeds were coated with 1% (w/v) CMC. These seeds were then submerged in bioinoculant and allowed to soak for 30 min at 120 rpm in a shaker incubator (Sarathambal *et al.*, 2021). As a control, seeds that were soaked in SDW without the bioinoculant were kept. To track seed germination and growth over 27 days, the seeds were sown in a seedling tray with sterile soil (Gupta and Van Staden, 2021). Four weeks after sowing, measurements of root and shoot length were made. These variables were used to contrast the sample plant's growth rate with the control plants (Kumar *et al.*, 2021). All tests were performed in triplicates and values are expressed as mean ± standard error. At $P < 0.05$, the one-way ANOVA with DMRT (Duncan Multiple Range Test) in IBM SPSS Statistics 21 was used to assess the significant difference.

Results and discussion

Isolation and identification of *B. albus* NJ01: Based on phenotypic characteristics 14 isolates were chosen for further plant growth study. The ability of bacteria to produce plant growth-promoting metabolites like IAA, siderophore and ammonia was determined using specific assays. The phosphate and zinc solubilisation ability of bacteria were also determined. After this the best isolate that showed positive results for all the assays was identified as gram-positive rods. It was sequenced to identify as *Bacillus albus* NJ01 strain with accession number OP784795 (Fig. 1).

Test for IAA production: IAA is an auxin that increases root and shoot length in plants by cell division, apical dominance, cell elongation, and tissue elongation. It also elongates epidermal root hairs (Jochum *et al.*, 2019). In the present study, *B. albus* NJ01 has shown 73.98 µg/mL of IAA. A study has reported that *Bacillus* spp. 3MK13 produced 25.81±0.88 µg/mL and other *Bacillus* spp ranging from 10.28 µg/mL to 21.08 µg/mL (Shah *et al.*, 2020). Another study on *B. subtilis* CW-S has reported that the strain produced 5.342 g/mL of IAA (Abuhena *et al.*, 2022).

Test for siderophore production: Siderophores are iron scavengers produced by bacteria. They chelate Fe^{3+} ions

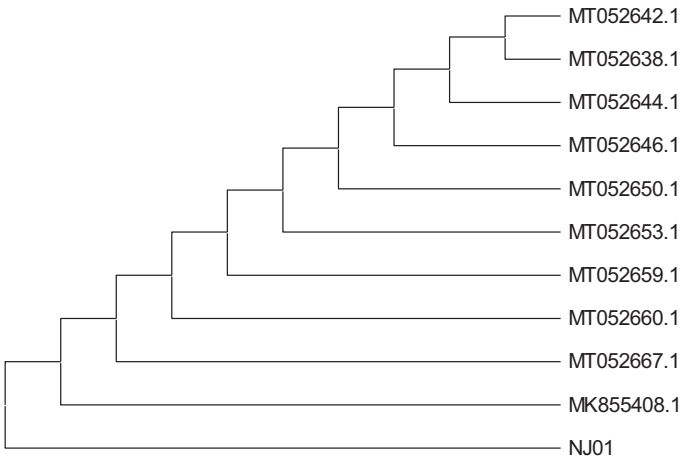

Fig. 1. Phylogenetic tree showing isolate NJ01

from the rhizosphere of the soil thereby hindering the growth of plant pathogens by reducing the amount of iron near the roots of plants (Moon and Ali 2022). A study highlights that siderophore production has been reported in *B. aerophilus* strain TR15c (Kumar *et al.*, 2021). Another study has confirmed the production of siderophores in *B. subtilis* NPROOT3 (Nalli *et al.*, 2023). Research has also reported the production of siderophores in *B. subtilis* (CWTS 5) (Chandwani *et al.*, 2023). In the current study, *B. albus* NJ01 has shown colour change from light yellow to brown confirming the production of siderophores (Fig. 2b).

Test for ammonia production: The most crucial crop nutrient, nitrogen, is bound by ammonia and made available for plants (Subramaniam *et al.*, 2016). Therefore, Ammonia production in trace amounts is considered an essential plant growth-promoting trait (*Sayyed et al.*, 2019). A study has reported ammonia production in *B. cereus* LPR2 as a plant growth-promoting trait (Kumar *et al.*, 2020). Another study has reported ammonia production in *B. amyloliquefaciens* (Xue *et al.*, 2021). Research has also reported the production of ammonia in *B. velezensis* CE 100 (Choub *et al.*, 2021). In the present study, *B.albus* NJ01 has shown ammonia production.

Test for phosphate solubilisation: By mineralizing organic phosphorus (P), solubilising inorganic phosphorus minerals, and storing significant amounts of phosphorus in biomass, phosphorus-solubilising microorganisms serve a crucial role in soil, a vast microflora that mediates bioavailable soil phosphorus (Nagrale *et al.*, 2023). Another study has reported phosphate solubilisation in *Bacillus* sp. MVY-004 (Mažylytė *et al.*, 2022). *B. atrophaeus* GQJK17 S8 has confirmed the solubilisation of phosphate (Mahdi *et al.*, 2021). In this study, *B.albus* NJ01 has shown a zone around the culture indicating phosphate solubilisation.

Test for zinc solubilisation: Inadequate supplies of zinc, a micronutrient important to numerous physiological processes in plants, will lower crop yields (Dinesh *et al.*, 2015). Therefore, zinc solubilisation by plant growth-promoting rhizobacteria is an important factor to improve plant growth (Maheshwari 2010). A zinc solubilising *B. subtilis* strain IA6 has been reported (Ahmad *et al.*, 2021). Similarly, another study has confirmed that *B. atrophaeus* GQJK17 S8 can solubilise zinc. (Mahdi *et al.*, 2021). Another research reported the zinc solubilising *Bacillus* spp PG-8 (Gohil *et al.*, 2022). In this study, *B. albus* NJ01 has shown a zone around the culture indicating zinc solubilisation.

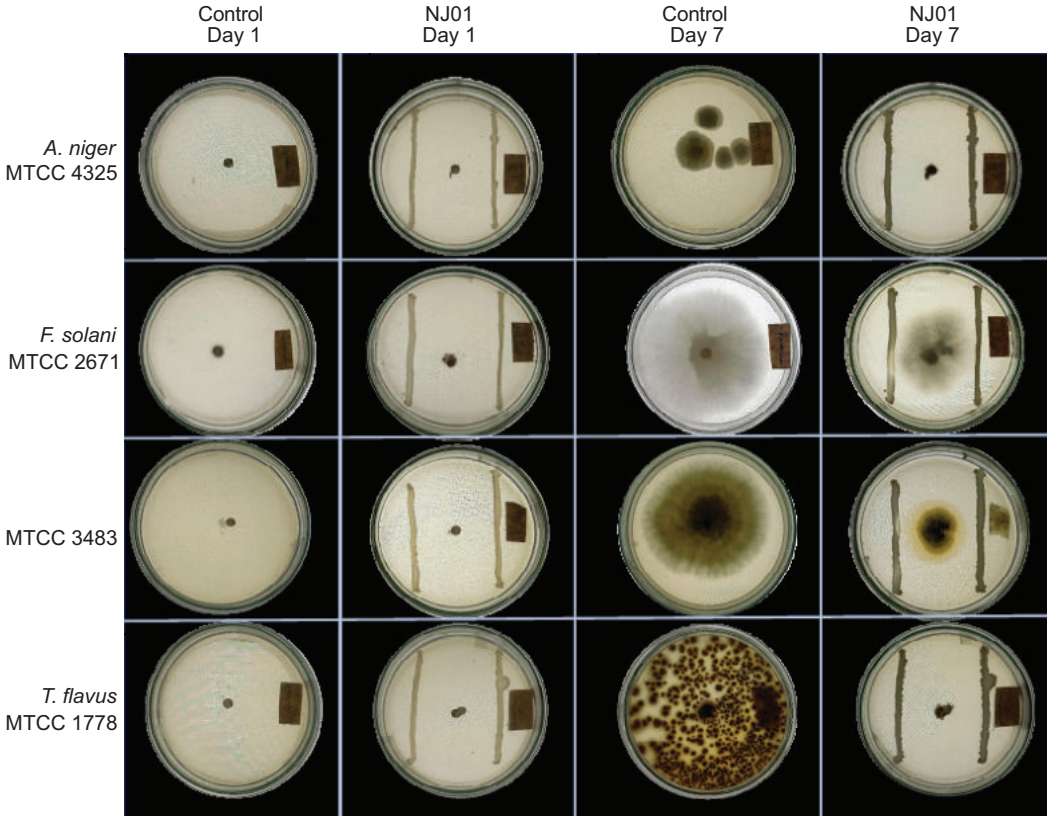

Fig. 2. Antifungal assay of *B. albus* NJ01 after 7th day of inculation

Antifungal assay: Antagonism against pathogenic fungi is a crucial trait of PGPR. *Fusarium* sp., known to cause *Fusarium* wilt disease, pose a significant threat to many plants, including tomatoes. Certain strains of *Cladosporium* function as saprophytic plant pathogens, affecting a wide range of plant hosts. In previous research, antifungal property of *B. subtilis* 30VD-1 strain against *Fusarium* sp. was reported (Khan *et al.*, 2018). *A. flavus* is recognized as an opportunistic pathogen, particularly detrimental to maize plants under drought or heat stress conditions. *Talaromyces* species, typically non-pathogenic to plants, have been investigated for their potential use as biofertilizers. Previous studies have demonstrated the antagonistic activity of *B. subtilis AU195* against *A. flavus* (Moyne *et al.*, 2001). In the present study, *B. albus* NJ01 demonstrated antagonism against four pathogenic fungi, namely *Talaromyces albobiverticillius, Cladosporium tenuissimum, Aspergillus niger, and Fusarium solani*, highlighting its capability to control phytopathogens (Fig. 2). This suggests that *B. albus* NJ01 is a robust candidate for managing fungal diseases in plants and promoting overall plant health.

Plant growth study on tomato: Seed germination test was done using tomato seeds of Arka Samrat variety. In the current study, the root length and shoot length of plants treated with *B. albus* NJ01 were found to be 7.98 ± 0.24 and 10.24 ± 0.11 cm respectively. And the root length and shoot length of control plants were found to be 5.58 ± 0.15 and 8.25 ± 0.82 cm respectively (Table 1, Fig. 3). Results show that the root and shoot length of tomato was improved when *Bacillus* spp. was used as a bioinoculant (Mengistie and Awlachew 2022). In another work it is observed that *B. safensis* strain SCAL1 helped tomato plants tolerate heat stress and improved the length of the root and stem (Mukhtar *et al.*, 2023). A work reported an increase in average lateral roots of tomato by 26% (Batista *et al.*, 2021).

B. albus NJ01 is a promising PGPR with IAA production that has been shown to significantly increase root length and shoot length in tomato plants after 30 days. The scope of the study can be extended to investigate the impact of *B. albus* NJ01 bioinoculant on tomato fruiting and its efficacy on other important crops. Additionally, its effects on plant growth under various stress conditions, such as salinity, heat, and drought, warrant further exploration. Moreover, *B. albus* NJ01 exhibits significant antifungal activity against several pathogenic fungi, including *Talaromyces albobiverticillius, Cladosporium tenuissimum, Aspergillus niger, and Fusarium solani*. The antagonism of *B. albus* NJ01 against *Fusarium oxysporum*, a harmful fungal pathogen of tomatoes, can be studied to further confirm its effectiveness in promoting healthy tomato growth. *B. albus* NJ01 also possesses phosphate and zinc solubilisation, siderophore

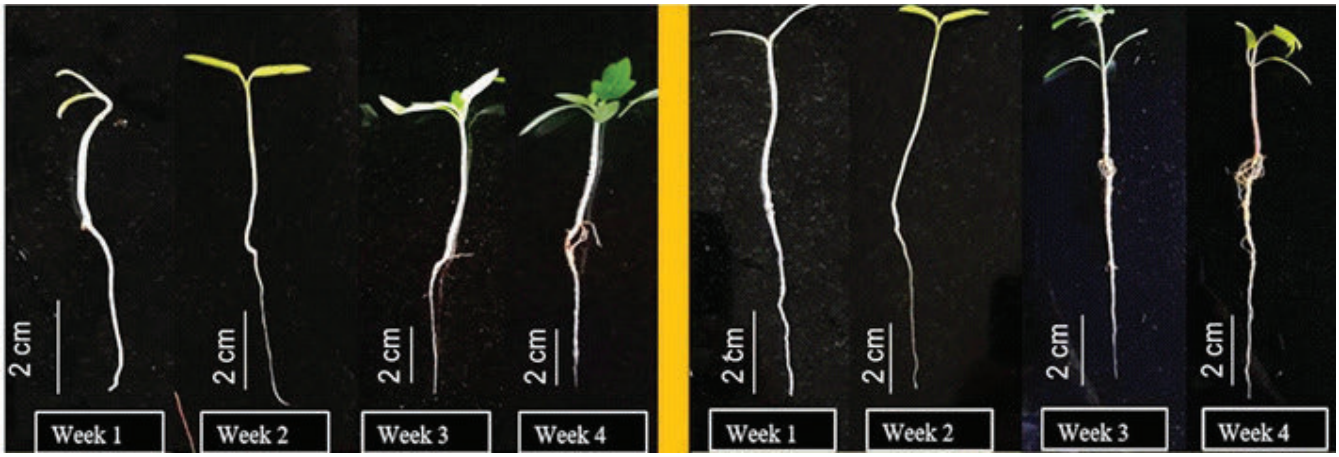

Fig. 3. Seedling growth of *S. lycopersicum* Control plants (left) and plants grown with *Bacillus albus* NJ01 (right)

production, and ammonia production. These attributes collectively contribute to the growth of healthier plants. Future research should focus on the bioinoculant's broader applications, including its impact on fruiting and its effectiveness against a wider range of crops and environmental stresses, to fully harness its potential in enhancing agricultural productivity.

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