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Unveiling the tripartite synergistic interaction of plant-arbuscular mycorrhizal fungus symbiosis by endophytic *Bacillus velezensis* S141 in *Lotus japonicus*

Sutee Kiddee[1] · Jenjira Wongdee[2] · Pongdet Piromyou[2] · Pongpan Songwattana[2] · Teerana Greetatorn[2] · Nantakorn Boonkerd[1] · Neung Teaumroong[1] · Katsuharu Saito[3] · Panlada Tittabutr[1]

Abstract

The crucial roles played by arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) in enhancing plant nutrient uptake and soil quality are widely recognized across various plant species. This study explored the effects and potential of *Bacillus velezensis* S141 as a plant growth-promoting rhizobacterium on promoting a symbiotic relationship of AMF, *Rhizophagus irregularis* with *Lotus japonicus*. *B. velezensis* S141 inoculation positively influenced fungal growth and development. *B. velezensis* S141 promoted fungal abundance, such as AM root colonization and spore number. It also boosted plant nutrient uptake, enhancing the nitrogen and phosphorus concentration by 1.65 and 1.51 times, respectively, under tripartite interaction conditions. However, the indole-3-acetic acid (IAA) producing capability of *B. velezensis* S141, based on the inoculation experiment test of S141 mutants defective in IAA synthesis, was not the key mechanism for promoting this symbiotic interaction. Interestingly, the S141 strain, originating from rhizospheric soil fields of soybeans, was found to penetrate plant root cells and establish itself as an endophyte. The presence of *B. velezensis* S141 not only triggered the expression of marker genes associated with early stages of AMF colonization and nutrient uptake in the host plant, but it also led to an upregulation of AMF genes responsible for cell cycle regulation. These results suggest that *B. velezensis* S141 holds promise as a helper bacterium in promoting plant-AMF symbiosis.

Keywords *Bacillus velezensis* S141 · *Lotus japonicus* · *Rhizophagus irregularis* · Symbiosis · Tripartite interaction

1 Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria that enhance plant growth and development (Oleńska et al. 2020; Vocciante et al. 2022; Bhat et al. 2023). These beneficial bacteria, including various species of *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Burkholderia,* and *Bacillus*, are found in association with plant roots (Figueiredo et al. 2010; Bashan et al. 2014; Singh et al. 2019), and some of them have been identified for their potential applications as biofertilizers (Sun et al. 2020; Barin et al. 2022). The growth-promoting effects contributed by these bacteria are mediated through diverse mechanisms, for example, nitrogen (N) fixation (Liu et al. 2019; Matse et al. 2019), phosphate solubilization (Prabhu et al. 2019; Amy et al. 2022), production of plant growth hormones, and biocontrol of plant pathogens (Odoh et al. 2019; Chauhan et al. 2021).

Arbuscular mycorrhizal fungi (AMF) significantly benefit plant growth through their symbiotic relationships with plant roots (Genre et al. 2018). They enhance plant resistance to environmental stress. They do so by helping in osmolyte accumulation and ion absorption and fostering soil fertility through the enhancement of water and mineral nutrition via their mycelial hyphae (Kempel et al. 2010; Mitra et al. 2021). The symbiotic relationship is established by a complex and sophisticated mechanism between AMF and the

✉ Panlada Tittabutr
  panlada@sut.ac.th

1   School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

2   Institute of Research and Development, Suranaree University of Technology, Muang, Nakhon Ratchasima 30000, Thailand

3   Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano 399-4598, Japan

host plants. The interaction begins with the release of signaling molecules, such as chitooligosaccharides and lipochitooligosaccharides from the AMF, which are then recognized by plant receptors on the root surface (Feng et al. 2019; Allen Rush et al. 2020). This recognition event activates symbiotic signaling pathways in the plant that result in the development of specialized fungal structures, called arbuscules, within the cortical cells of the plant root. Arbuscules contribute to the supply of nutrients, such as phosphorus (P) and N, to the plant (Smith 1988; George et al. 1995; Smith et al. 2011).

In the rhizosphere, synergistic interactions between PGPR and AMF have been observed to enhance plant growth and performance through an increase in AMF colonization, regulation of plant hormones, such as auxin and cytokinin, and inhibition of plant pathogens (Tsukanova et al. 2017; Emmanuel & Babalola 2020). For example, co-inoculation of soybean with *B. amyloliquefaciens* and AMF led to an increase in AMF colonization, which is postulated to have contributed to improvements in plant growth and field yield (Sheteiwy et al. 2021). Our previous research also confirms that the combined application of PGPR *Brevibacillus* sp. SUT47 with AMF (*Acaulospora tuberculata*) resulted in increased AMF colonization and spore production in maize. This was in comparison to single AMF inoculation in sterilized sand, which was conducted under laboratory conditions (Yuttavanichakul et al. 2018; Kiddee et al. 2020). Recently, Begum et al. (2022) showed that *Bacillus methylotrophicus* co-inoculated with AMF produced an increase in photosynthetic activity and mineral, osmolyte, and phytohormone content in tobacco in the field. These increases positively affected secondary metabolite content and antioxidant system under drought stress conditions and resulted in enhanced tobacco growth.

B. velezensis is a recently characterized PGPR species that has shown potential as a biocontrol agent (Adeniji et al. 2019; Rabbee et al. 2019). For example, *B. velezensis* FZB42 has been reported to produce secondary metabolites with antimicrobial properties (Fan et al. 2018). Yet another strain, *B. velezensis* S141, efficiently mitigated cercospora leaf spots in mung beans when isolated from soybean-adjoining rhizospheric soil in Thailand and sprayed on leaf surfaces (Prakamhang et al. 2015; Songwattana et al. 2023). In soybean-*Bradyrhizobium* symbiosis, *B. velezensis* S141 colonized the nodule and root surface and induced the production of larger nodules and enhanced nitrogen-fixing efficiency by *B. diazoefficiens* USDA110 (Sibponkrung et al. 2020). *B. velezensis* S141 has a set of genes for auxin biosynthesis in its genome (Sibponkrung et al. 2017). After inoculating the disrupted strains of those genes into soybean-*Bradyrhizobium*, the disrupted of auxin biosynthesis attenuated the effects of *B. velezensis* S141 on increasing nodule size and promotion of N-fixing activity. These findings suggest that

this specific PGPR strain may play an important role in enhancing the symbiotic functionality between rhizobia and legumes through its phytohormone producing properties.

In contrast, it remains to be elucidated whether *B. velezensis* S141 exerts an influence on plant–AMF symbiosis. Phytohormones, including auxins, have been shown to modulate the development of the mycorrhizal symbiosis (Gutjahr 2014; Pozo et al. 2015; Liao et al. 2018). Etemadi et al. (2014) demonstrated that overexpression of miR393, an miRNA that specifically targets a plant auxin receptor gene, resulted in suppression of arbuscule formation, suggesting a necessary role for auxin perception in arbuscule development. In this situation, we hypothesized that *B. velezensis* S141 could affect the symbiotic relationship between plants and AMF through its potential to produce auxins. To test this hypothesis, we investigated the effects of *B. velezensis* S141 and its mutant strains containing disrupted auxin biosynthesis genes on AMF development both intra- and extra-radically. Since the interactions between *L. japonicus* and *R. irregularis* has been intensively studied in the molecular level due to the available of genome information, *L. japonicus* and *R. irregularis* were selected as a model to investigate the effect of *B. velezensis* S141 on promoting plant-AMF symbiosis. In addition, we analyzed the gene expression of symbiosis-related genes in both AMF and host plant.

2 Materials and methods

2.1 Biological materials and growth conditions

L. japonicus B-129 seeds were surface-sterilized with a 3% (w/v) sodium hypochlorite solution for 10 min and then thoroughly rinsed with sterile distilled water. The sterilized seeds were germinated on moist paper in a Petri dish and incubated at 26 °C in the dark for 2 days followed by 3 more days under light. Seedlings were transplanted into 50-mL centrifuge tubes (one plant per tube), each with a drainage cavity at the bottom, which contained filter paper with a pore size of 2 μm and filled with autoclaved river sand (particle size, 0.5–2.0 mm) as planting material. This filter allows the bacteria to remain in the system while allowing water to flow out. The sand was washed several times until the water ran clear and sterilized at 121 °C for 45 min by autoclaving and repeating the sterilization twice within 24 h. The concentration of commercial AMF inoculum *R. irregularis* DAOM197198 (Mycorise, Premier Tech, Rivière-du-Loup, Canada) at 4,000 spores/mL was diluted using sterilized water to achieve a final spore concentration of 250 spores/mL. Subsequently, a hole was carefully made at the center of the pot containing planting material with sufficient depth to cover the roots of *L. japonicus*. The AMF spores were then

evenly distributed around the hole, followed by transplanting the plant and covering it with river sand.

The plant growth-promoting rhizobacterium *Bacillus velezensis* S141 (Sibponkrung et al. 2017) was cultured in Luria–Bertani (LB) broth at 28 °C for 20 h and used as inoculum. Derivative mutant strains of *B. velezensis* S141 (Δ*IPyAD*, Δ*yhcX* and Δ*dhaS*), which have a reduced production of indole-3-acetic acid (IAA), were cultured under the same conditions but in the presence of antibiotics [1 μg/mL mg erythromycin, 10 μg/mL kanamycin, and 100 μg/mL spectinomycin] to ensure that only the desired bacteria were grown, while the *B. velezensis* S141 GFP-tagged strain was cultured in LB medium supplemented with 8 μg/mL phleomycin (Sibponkrung et al. 2020). Bacterial cells were separated by centrifugation and washed twice with 0.85% (w/v) NaCl. The cell density was then adjusted to $OD_{600} = 1$ (approximately 10^8 CFU/mL). The bacterial cells at different cell concentrations (10^5, 10^6, and 10^7 CFU in 50 mL) were then inoculated or co-inoculated with *R. irregularis* in sand. Non-inoculated control plants were also included in the experiment by adding them with 50 mL of 0.85% (w/v) NaCl.

Plants were grown in a growth chamber at 28 °C under a 16/8-h light and dark cycle and a light intensity of 150 μmol m^{-2} s^{-1}. The plants were supplied with a half-strength Hoagland's solution containing a low concentration of KH_2PO_4 (100 μM) every 2 days since high P concentration could inhibit AMF infection (Sugimura and Saito 2017). Plants were harvested at either 45 or 60 days after inoculation (DAI), depending on the specific data collection goals, such as assessing plant growth, nutrient uptake, colonization efficiency, spore count, or the localization of arbuscular mycorrhizal fungi (AMF) or bacteria. Each experiment was conducted with triplicate samples. For plant growth data collection, the plants were harvested and separated into shoot and root components. Subsequently, these plant parts were dried at 70 °C for 48 h and weighed following the method outlined by Shipley and Vu (2002). Plant height above ground level was measured at 45 or 60 DAI, as specified in each experiment. Chlorophyll content in fully expanded leaves from the top was estimated in SPAD units using the SPAD-502 Plus instrument (Konica Minolta Optics, Japan). Each treatment was replicated four times in the experiment.

2.2 Analysis of plant P and N contents

Plant shoots were dried at 70 °C for 48 h, finely ground, and weighed. Subsequent analysis of phosphorus (P) involved acid digestion in a solution of nitric and perchloric acids ($HNO_3 + HClO_4$). P concentration was then established using the vanadomolybdate blue method (Watanabe and Olsen 1965). Pursuing the nitrogen (N) analysis, plant samples were heated in a Kjeldahl digestion apparatus,

gradually increasing the temperature to 200 °C, then further to 350–375 °C for 1 h until transparent. N concentrations were ascertained using the Kjeldahl method (Yash and Kalra 1998).

2.3 Localization of bacteria in roots

L. japonicus were inoculated with *B. velezensis* S141 at a concentration of 10^5 CFU/plant or co-inoculated with both *B. velezensis* S141 and 250 spores/plant of *R. irregularis*. These plants were grown under the conditions described above. At 7, 15, 30 and 45 DAI, roots were harvested and thoroughly washed with sterilized water to remove sand particles. These roots were then cut into fine segments, each 1–2 cm in length. The segments were embedded in 5% agarose gel and sectioned into 40 μm thick slices using a Leica vibratome (VT1000S, Leica Microsystems, Germany). The sections were stained for 10 min with 5 μM SYTO 9 (Thermo Fisher Scientific, USA) to visualize bacterial cells and 0.01% (w/v) Calcofluor (Bonaldi et al. 2011) to emphasize plant roots and AMF cell walls. We did not use the GFP-tagged strain in this study due to low fluorescence expression. However, the experiment was conducted under sterile conditions, enabling comparisons with the non-inoculated control. No bacterial cells or fungus were detected under the microscope in the control group, eliminating the possibility of seed endophyte contamination. The stained sections were washed with phosphate-buffered saline (PBS), mounted on a glass slide with 10% PBS-glycerol solution, and covered with a coverslip. The Calcofluor was excited at 405 nm, and its emission signal was detected using a 460–500 nm filter for image acquisition. SYTO 9 fluorescence was detected by exciting the samples with a 488 nm laser line and collecting the emission signal at 490 to 522 nm. Bacterial localization was observed using a Nikon inverted Eclipse Ti-E Confocal Laser Scanning Microscope (Nikon, Japan).

2.4 Enumeration of endophytic bacteria

The roots of *L. japonicus*, inoculated with and without the S141 GFP-tagged strain (Sibponkrung et al. 2020), were surface-sterilized 30 days after infection. This process involved immersing the roots in a 3% NaClO solution for 5 min and then soaking them in 70% ethanol for another 5 min. Once sterilized, the roots were thoroughly rinsed at least five times with sterile distilled water (Pongdet et al. 2015). The sterilized plant roots were then ground with 2 mL of 0.85% NaCl. Serial dilutions were prepared from this mixture, ranging from 10^0 to 10^4. From each dilution, 100 μL was plated onto LB agar, which was augmented with 8 μg/mL of phleomycin. The plates were then incubated at a temperature of 28 °C for a period of 20 h to allow for the enumeration of the target

strain. The number of endophytic bacteria present was calculated as CFU/plant.

2.5 Assessment of AMF colonization

The root system of *L. japonicus* was collected and carefully cleaned by removing all soil particles. The cleaned roots were cut into 1–2 cm segments and washed with tap water. To remove the cytoplasmic content of the plant root cells, these root segments were treated with 10% (w/v) potassium hydroxide (KOH) at 90 °C for 10 min followed by acidification with 2% (v/v) hydrochloride acid (HCl) for 5 min. The segments were subsequently stained with 0.05% trypan blue in lactic acid at 90 °C for 10 min. The degree of AMF colonization was assessed using the method described by Trouvelot et al. (1986) with some modifications. Briefly, approximately 10 root fragments from each plant were mounted on glass slides and observed under a light microscope with a $10\times$ objective. Each slide was observed over a total of 100 microscopic fields. The intensity of AMF colonization and the abundance of arbuscules in a field of view were categorized into six and three classes, respectively. Based on the scores obtained from 100 fields of view, five parameters of AMF colonization (F%, M%, m%, A%, and a%) were calculated according to Trouvelot et al. (1986). F% represents the frequency of mycorrhiza in the root system, M% represents the intensity of the mycorrhizal colonization in the root system, m% represents the intensity of the mycorrhizal colonization in the root fragments, A% represents the arbuscule abundance in the root system, and a% represents the arbuscule abundance in the mycorrhizal parts of the root fragments.

2.6 Quantification of AMF in roots by quantitative PCR

Inoculated root samples of *L. japonicus* were collected, and total DNA was extracted using the Dneasy® Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The abundance of fungi was estimated through quantitative real-time polymerase chain reactions (qPCR), using approximately 50 ng of each DNA sample on a StepOne Real-Time PCR System (Thermo Fisher Scientific). Four biological replicates were performed in this experiment. A primer set specific for *R. irregularis* was used for qPCR. The set consisted of a forward primer (CCCACCAGGGCAGATTAATC), a reverse primer (TGGCTTTGTACAGGCAACAG), and a TaqMan-MGB probe (197198P: FAM-CCCTGGAGTATCTG-MGBEQ synthesized by Eurofins Genomics, Japan). This primer set was designed based on the single-copy *cox3-rnl* intergenic region in mitochondrial DNA (Badri et al. 2016). As an internal control, a primer set designed for genomic DNA

of the *L. japonicus* ubiquitin-conjugating enzyme E2 gene (LotjaGi1g1v0401300.1) was used. The primer set for this gene consisted of a forward primer (AAATGGACGGCTCTTATCAAGGT), a reverse primer (GACCGGTCGAACATCTTACACA), and a TaqMan-MGB probe (FAM-TGCTGGCTAATATGC-MGBEQ). Data were normalized relative to this internal control and analyzed according to the $2^{-\Delta\Delta Ct}$ method with four replications (Livak and Schmittgen 2001).

2.7 Enumeration of *R. irregularis* spores in soil

AMF spores were extracted by wet sieving and decanting (Gerdemann and Nicolson 1963; Daniels and Skipper 1982). All soil contained in a 50 mL tube was collected and mixed with 100 mL of tap water in a 500 mL beaker. The soil sample was agitated vigorously to disperse the AMF spores and left to stand for 5 min to allow the heavier soil particles to settle to the bottom. The supernatant was decanted through standard sieves with pore sizes of 250, 106, 75, and 38 μm. The AMF spores and soil particles retained on the 106, 75, and 38 μm sieves were collected in 15 mL centrifuge tubes and centrifuged at 4,000 rpm for 5 min. After removing the supernatant, 40% (w/v) sucrose solution was added and centrifuged at 5,000 rpm for 5 min. The supernatant was carefully removed and poured into sieves to retain the AMF spores. The spores on the sieves were rinsed with tap water and collected in Petri dishes. AMF spores were counted under a stereomicroscope.

2.8 Gene expression analysis

Total RNA was extracted using RNA Prep Pure Plant Plus Kit (TIANGEN Biotech, China). To remove DNA contamination, the RNA samples were treated with Dnase I (Thermo Fisher Scientific). cDNA was synthesized from the RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Quantitative RT-PCR was conducted using Luna Universal qPCR Master Mix (New England Biolabs, USA) and the CFX Opus 96 Real-Time PCR System (Bio-Rad, USA). The primers for this system are listed in Supplementary Tables S1 and S2. As an internal control for gene expression, we used elongation factor 2 (*EF2*) in *L. japonicus* and *EF1β* in *R. irregularis* (Kobae et al. 2015). We evaluated three biological samples of the plant at 45 DAI due to resource limitations and experimental practicality, each using approximately 5 ng of cDNA template. We calculated relative gene expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

2.9　Statistical analyses

We conducted statistical analyses using R software (version 4.2.2). To identify specific differences among samples belonging to three or more groups, we used Tukey's honestly significant difference (HSD) test whenever ANOVA pointed to a significant discrepancy in group means. We also applied the Levene test to verify normal distribution and demonstrate equal variances across the groups. To compare the effects of single inoculation with *R. irregularis* against co-inoculation with *R. irregularis* and *B. velezensis* S141, we used Student's *t*-test. We visualized all data using GraphPad Prism (v.9.1.1).

3　Results

3.1　*B. velezensis* S141 enhances AMF abundance in the interior and exterior of *L. japonicus* roots

To investigate whether *B. velezensis* S141 could enhance the development of *R. irregularis* during its symbiosis with *L. japonicus*, we assessed AMF abundance in roots and quantified spore production in soil. We co-inoculated AMF with *B. velezensis* S141 using different cell densities on *L. japonicus* and measured the extent of AMF colonization at 60 DAI. The presence of *B. velezensis* S141 caused a slight but significant increase in the intensity of AMF colonization (M% and m%) compared to the absence of *B. velezensis* S141 (Fig. 1A). This enhancement was consistently observed across all *B. velezensis* S141 inoculum

cell densities. In contrast, the frequency of AMF colonization (F%) and arbuscular abundances (A% and a%) were not affected by inoculation with *B. velezensis* S141. AMF abundance in roots was further quantified using a hydrolysis probe-based qPCR assay specifically targeting *R. irregularis* DAOM197198 mitochondrial DNA as described by Badri et al. (2016). The relative copy number of the *cox3-rnl* intergenic region consistently increased more than twofold in the co-inoculation treatments compared to AMF single inoculation (Fig. 1B). Notably, *B. velezensis* S141 also promoted AMF spore production in soil (Fig. 1C). Regarding the inoculum cell density of *B. velezensis* S141 at 10^5, 10^6, and 10^7 CFU/plant, the co-inoculation produced an increase in the number of spores produced in soil by 39%, 19%, and 27%, respectively when compared to single inoculation with *R. irregularis*. These results show that *B. velezensis* S141 significantly promoted AMF development both inside and outside the roots.

3.2　*B. velezensis* S141 affects mycorrhizal plant growth

To determine the direct effect of *B. velezensis* S141 on *L. japonicus* growth, the plant was inoculated with different densities of the bacteria in the absence of AMF. At 60 DAI, *B. velezensis* S141 did not affect the plant growth of *L. japonicus*. However, the bacterial cells at a density of 10^7 CFU/plant produce a significant increase in the shoots' dry weights by 2.1-fold compared to the non-inoculated plants (Table S3). Although inoculation only AMF did not notably enhance plant growth at day 60 compared to non-inoculated
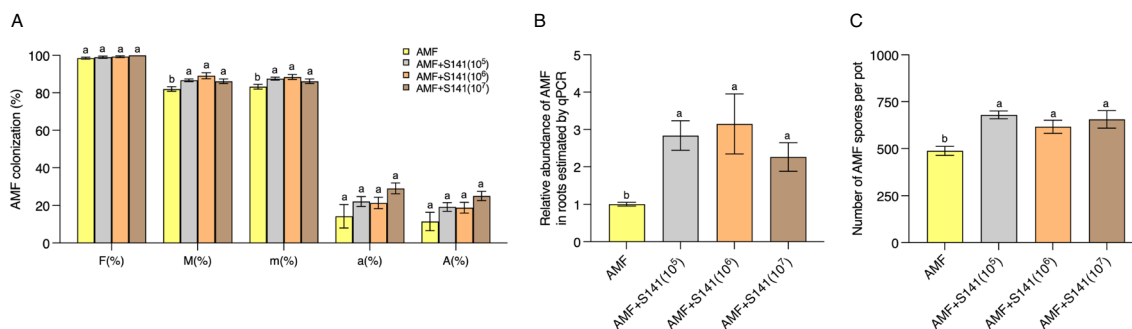
Fig. 1 The abundance of *R. irregularis* at 60 days after inoculation (DAI). *L. japonicus* was either inoculated with *R. irregularis* alone (AMF) or co-inoculated with both *R. irregularis* and *B. velezensis* S141 at different cell densities: 10^5 (AMF+S141(10^5)), 10^6 (AMF+S141(10^6)), 10^7 (AMF+S141(10^7)) CFU/plant. (**A**) AMF colonization in *L. japonicus* roots. F(%), the frequency of mycorrhiza in the root system; M(%), the intensity of the mycorrhizal colonization in the root system; m(%), the intensity of the mycorrhizal colonization in the root fragments; a(%), the arbuscule abundance in the mycorrhizal parts of the root fragments; and A(%), the arbuscule abundance in the root system. (**B**) The relative abundance of *R. irreg-*

ularis in *L. japonicus* roots estimated by quantitative PCR (qPCR) using a hydrolysis probe designed from the *cox3-rnl* intergenic region in *R. irregularis* mitochondrial DNA. The abundance of *R. irregularis* in roots is normalized based on the copy number of the genomic region of the *L. japonicus* ubiquitin-conjugating enzyme E2 gene and expressed relative to that in plants singly inoculated with *R. irregularis* (AMF). (**C**) The number of *R. irregularis* spores produced in the soil. Values are means ± s.e.m. (*n*=4). Bars marked with the same letter are not significantly different at *p* < 0.05 on Tukey's HSD test. None of AMF were detected in the non-inoculated control plant

plants, when *B. velezensis* S141 was paired with AMF at a density of 10^5 CFU/plant, there was a significant rise in plant height compared to just AMF inoculation.

The 60-day growth period was probably too long to evaluate *L. japonicus* growth in a 50-mL pot. Therefore, we assessed plant growth at 45 DAI. A density of 10^5 CFU/plant was selected for inoculation with *B. velezensis* S141, a density that was shown to be effective in enhancing plant height at 60 DAI (Table S3), and it is the lowest density that significantly promote AMF spore production (Fig. 1C). Even at 45 DAI, a modest increase in AMF colonization, M% and m%, due to co-inoculation (Fig. 2A) was found. No direct effect of S141 on *L. japonicus* growth was observed at 45 DAI (Fig. 2B, C). Conversely, the effect of AMF inoculation was pronounced, namely plant heights and dry weights of AMF-inoculated plants were significantly greater than those of non-inoculated plants. In particular, the co-inoculation of *B. velezensis* S141 and *R. irregularis* led to a maximal shoot dry weight approximately 1.2-fold higher than that of the single AMF inoculation (Fig. 2C). In addition, shoot nitrogen (N) and phosphorus (P) concentrations significantly increased with co-inoculation yielding 1.7- and 1.5-fold increases in N and P, respectively, relative to the single AMF inoculation (Fig. 2D). These results indicate that *B. velezensis* S141 promotes plant growth and nutrient acquisition when AMF is present.

3.3 *B. velezensis* S141 stimulates root elongation at early growth stage

To explore the possibility of a direct growth-promoting effect from *B. velezensis* S141 on *L. japonicus* during the early growth stages, we assessed shoot and root elongation at 7 and 15 DAI. *B. velezensis* S141 produced no influence on shoot elongation (Fig. 3). However, the length of tap roots inoculated with the bacteria was approximately double than that of non-inoculated plants at 15 DAI.
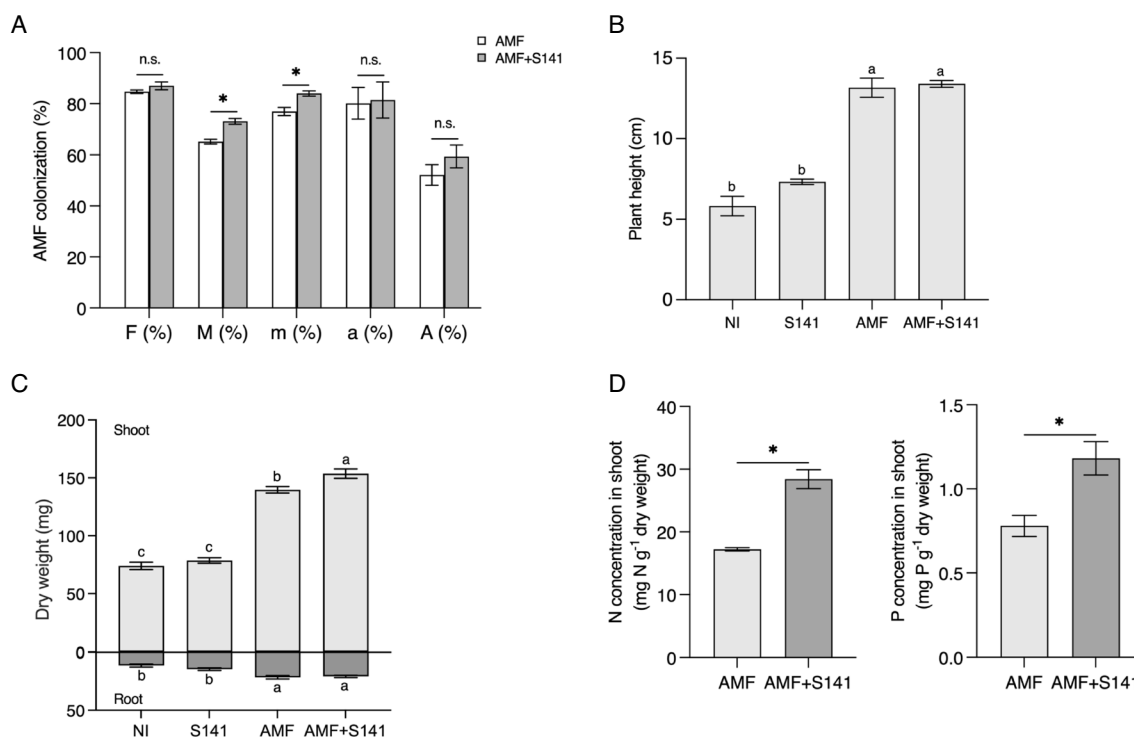
Fig. 2 *R. irregularis* abundance and *L. japonicus* growth at 45 days after inoculation (DAI) under four inoculation conditions: non-inoculation (NI), inoculation with *B. velezensis* S141 (S141), inoculation with *R. irregularis* (AMF), and co-inoculation with both *R. irregularis* and *B. velezensis* S141 (AMF+S141). Cell density of *B. velezensis* S141 was 10^5 CFU/plant. (**A**) AMF colonization in *L. japonicus* roots. F(%), the frequency of mycorrhiza in the root system; M(%), the intensity of the mycorrhizal colonization in the root system; m(%), the intensity of the mycorrhizal colonization in the root frag- ments; a(%), the arbuscule abundance in the mycorrhizal parts of the root fragments; and A(%), the arbuscule abundance in the root sys- tem. Plant height (**B**) and dry weight (**C**) of *L. japonicus*. (**D**) The concentration of nitrogen (N) and phosphorus (P) in *L. japonicus* shoot. The values presented are means ± s.e.m. ($n = 3$). Student's t-test (n.s., not significant; *, $p < 0.05$) was conducted in (**A**) and (**D**). Bars marked with the same letter are not significantly different at $p < 0.05$ on Tukey's HSD test in (**B**) and (**C**)

A

B

C
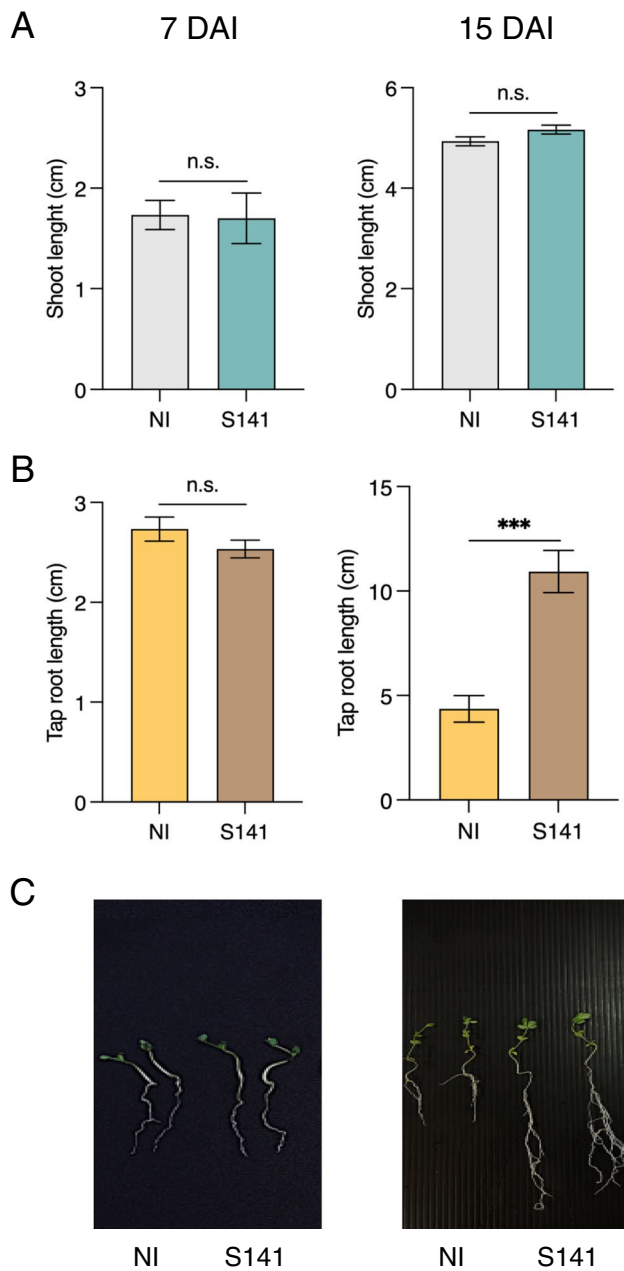
Fig. 3 Growth of *L. japonicus* inoculated with 10^5 CFU/plant of *B. velezensis* S141 (S141) or not inoculated (NI) at 7 and 15 days after inoculation (DAI). Graphs show shoot length (**A**) and tap root length (**B**). Values are means $\pm$ s.e.m. ($n = 4$). The statistical analysis was performed using Student's *t*-test (n.s., not significant; ***, $p < 0.001$). (**C**) Appearance of *L. japonicus* roots. Scale bars: 1 cm

3.4 IAA production-related genes in *B. velezensis* S141 are not involved in promoting tap root elongation and AMF colonization

Given that *B. velezensis* S141 produces IAA (Sibponkrung et al. 2020), and this IAA could stimulate root elongation in *L. japonicus* and consequently, AMF colonization.

Therefore, we investigated whether the IAA produced by *B. velezensis* S141 supported root elongation using derivative mutants of *B. velezensis* S141 (*ΔdhaS*, *ΔyhcX*, and *ΔIPyAD*) that yielded lower amounts of IAA than the wild type (Sibponkrung et al. 2020). The lengths of shoot and tap root of the plants that were inoculated with these mutant strains were comparable to those of plants inoculated with the wild type at both 7 and 15 DAI, indicating that the *B. velezensis* S141 *dhaS*, *yhcX*, and *IPyAD* genes do not significantly affect root elongation (Fig. 4A, B). In addition, we evaluated the abundance of AMF when co-inoculated with these mutant strains. We observed no significant differences in AMF colonization between inoculations of the wild type and mutant strains at 60 DAI (Fig. 4C). Overall, IAA produced by *dhaS*, *yhcX*, and *IPyAD* of *B. velezensis* S141 does not appear to be a major factor in promoting tap root elongation and AMF colonization in *L. japonicus*.

3.5 *B. velezensis* S141 affects gene expression in both *L. japonicus* and *R. irregularis* during symbiosis

We investigated the changes in gene expression associated with the symbiotic response of *L. japonicus* and *R. irregularis* after inoculation with *B. velezensis* S141. *L. japonicus* marker genes involved in the regulation of AMF accommodation within roots and nutrient exchange were assessed at 45 DAI. The expression of the subtilisin-like serine protease gene, *SbtM1*, which participates in the early phases of AMF colonization (Takeda et al. 2009), was enhanced sixfold after co-inoculation with *B. velezensis* S141 compared to the single AMF inoculation (Fig. S2A). The expression of the transcription factor gene *RAM1*, which is mainly responsible for arbuscule formation, was doubled. The mycorrhiza-specific H+-ATPase *HA1*, the phosphate transporter (*PT4*) and the ammonium transporter (*AMT2;2*), all of which are involved in P and N uptake in arbuscule-containing cortical cells (Javot et al. 2007; Guether et al. 2009; Krajinski et al. 2014; Wang et al. 2014), showed elevated expression levels in the presence of *B. velezensis* S141. For genes implicated in fatty acid biosynthesis and lipid transport in arbuscular mycorrhizal symbiosis, *B. velezensis* S141 produced enhancement of expression of the glycerol-3-phosphate acyltransferase gene *RAM2* (Wang et al. 2012) while the acyl-ACP thioesterase *FatM* (Bravo et al. 2017; Brands et al. 2018) and the ABC transporter gene *STR* (Zhang et al. 2010) did not undergo enhancement.

We also analyzed transcript levels of cell cycle-related genes and phophorus (P) and carbon (C) nutrition-related genes in *R. irregularis* colonizing *L. japonicus* roots. Five out of the seven cell cycle-related genes tested showed significantly higher expression
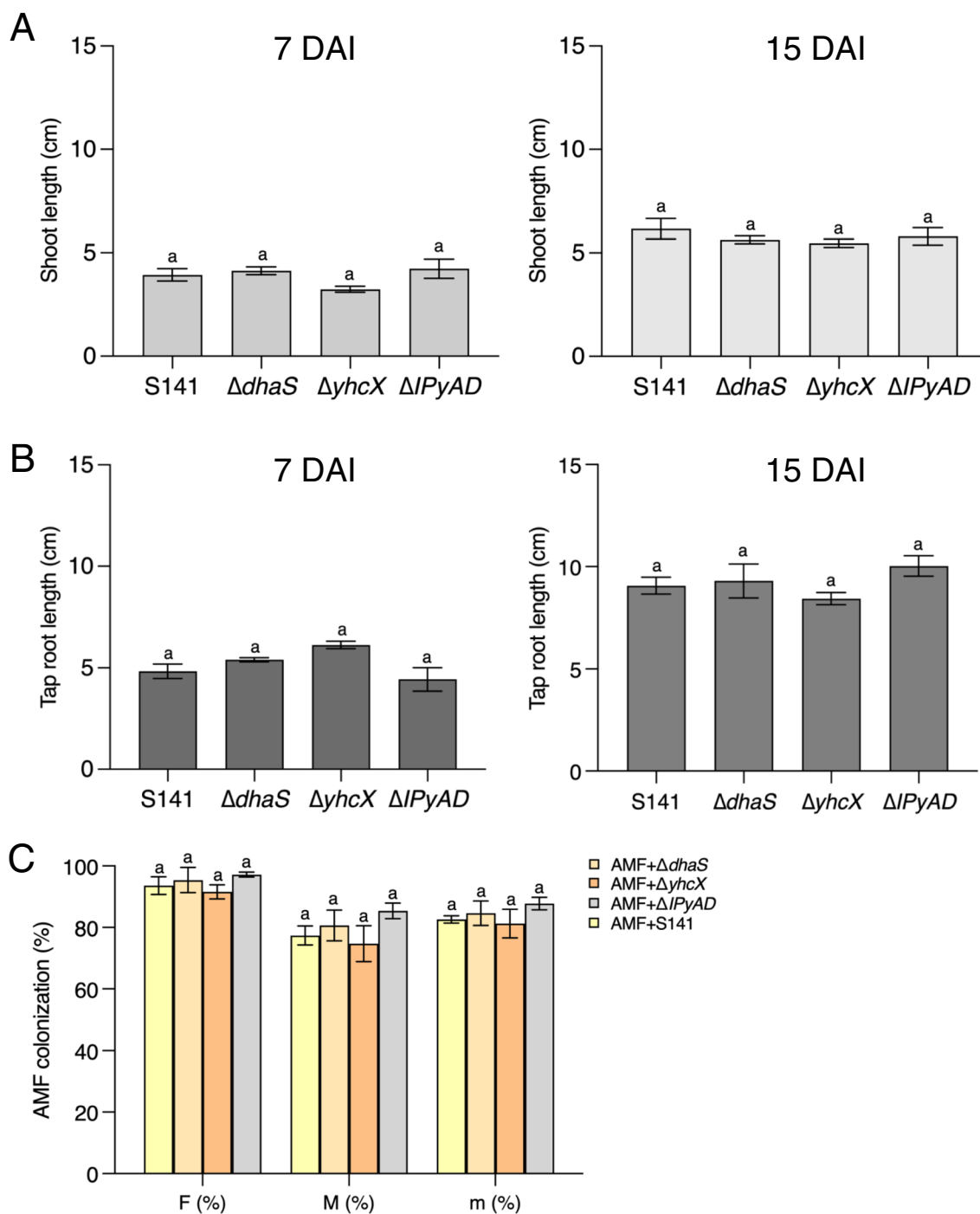
Fig. 4 The growth promotion of *L. japonicus* at 7 (**A**) and 15 (**B**) days after inoculation (DAI) when inoculation with *B. velezensis* S141 wide type (S141) and its derivative mutants (Δ*dhaS*, Δ*yhcX*, and Δ*IPyAD*) decreases in the indole-3-acetic acid (IAA) production. (**C**) The measurement of AMF colonization at 60 DAI (AMF root colonization was defined as F(%), the frequency of mycorrhiza in the root system, M(%), the intensity of the mycorrhizal colonization in the root system, and m(%), the intensity of the mycorrhizal colonization in the root fragments). Values are means ± s.e.m. ($n = 4$). Bars marked with the same letter are not significantly different at $p < 0.05$ on Tukey's HSD test

in roots with *B. velezensis* S141 than in those without the bacteria (Fig. S2B). For genes involved in P nutrition in *R. irregularis*, we focused on those related to polyphosphate synthesis and degradation (Ezawa and Saito 2018). The expression levels of genes encoding the polyphosphate-synthesizing enzymes, VTC1, VTC2,

and VTC4 (Nguyen et al. 2022), were found to have decreased upon co-inoculation with *B. velezensis* S141. On the other hand, the transcript levels of the endopolyphosphatase genes *PPN1–4* (Nguyen & Saito 2021) showed no significant differences. The expression of the monosaccharide transporter gene *MST2*, which plays a crucial role in sugar uptake in AMF, was reduced in the presence of *B. velezensis* S141.

3.6 *B. velezensis* S141 is an endophytic bacterium

The localization of *B. velezensis* S141 was examined using confocal microscopy to confirm the presence of the bacteria in *L. japonicus* roots during the symbiotic association with AMF. No bacterial cells were observed in the longitudinal root sections of non-inoculated plants at 7 and 45 DAI (Fig. 5A, C). Intriguingly, bacterial cells were observed within root cells at 7 DAI (Fig. 5B) although they did not colonize within intraradical hyphae and arbuscules of AMF (Fig. 5D). The amount of living endophytic bacteria was detected at 30 DAI at a density of approximately in log10 value as 2.15 ± 0.7 and 1.9 ± 0.6 CFU/plant in mycorrhizal and non-mycorrhizal plant roots, respectively, using a plate count technique.

4 Discussion

PGPRs and AMF can coexist in roots, and certain combinations of these microorganisms produce enhancement of both AMF abundance and plant growth (Meyer & Linderman 1986; Yuttavanichakul et al. 2018; Kiddee et al. 2020; Sheteiwy et al. 2021). In this study, we demonstrated that the endophytic PGPR *B. velezensis* S141 produced an increase in AMF colonization in *L. japonicus* roots and promoted spore production (Fig. 1). Although *B. velezensis* S141 was originally isolated as a PGPR for soybean, cell concentrations of 10^5 and 10^6 cells/plant showed no effect on the biomass production of *L. japonicus*. However, in the presence of AMF, *B. velezensis* S141 augmented the plant biomass in addition to shoot N and P concentrations (Fig. 2). This finding suggests that *B. velezensis* S141 can exert its PGPR effect in *L. japonicus* when coexisting with AMF. At 15 days after inoculation with *B. velezensis* S141, the root length more than doubled compared to that without the bacterial strain (Fig. 3). Similar plant growth promotion at the seedling stage has also been observed in peppers inoculated with *B. velezensis* BBC047 (Stoll et al. 2021). This accelerated initial root elongation may cause enlargement of the zone of AMF colonization in the early phase, leading to greater AMF abundance in roots. The increase in AMF colonization

Fig. 5 Confocal laser scanning microscopy images illustrating the bacterial localization in *L. japonicus* roots at 7 (**A**, **B**) and 45 (**C**, **D**) days after-inoculation (DAI). (**A, C**) Single inoculation of *R. irregularis*. (**B, D**) Co-inoculation of *R. irregularis* with *B. velezensis* S141. Longitudinal sections depict mycorrhizal roots stained with calcofluor (blue) to visualize cell walls of plants and arbuscular mycorrhizal fungi, and with SYTO 9 (green) to detect live bacterial cells (white arrowheads). Arbuscules of *R. irregularis* are highlighted by the yellow arrowhead. Scale bars denote 10 μm

AMF

AMF+S141
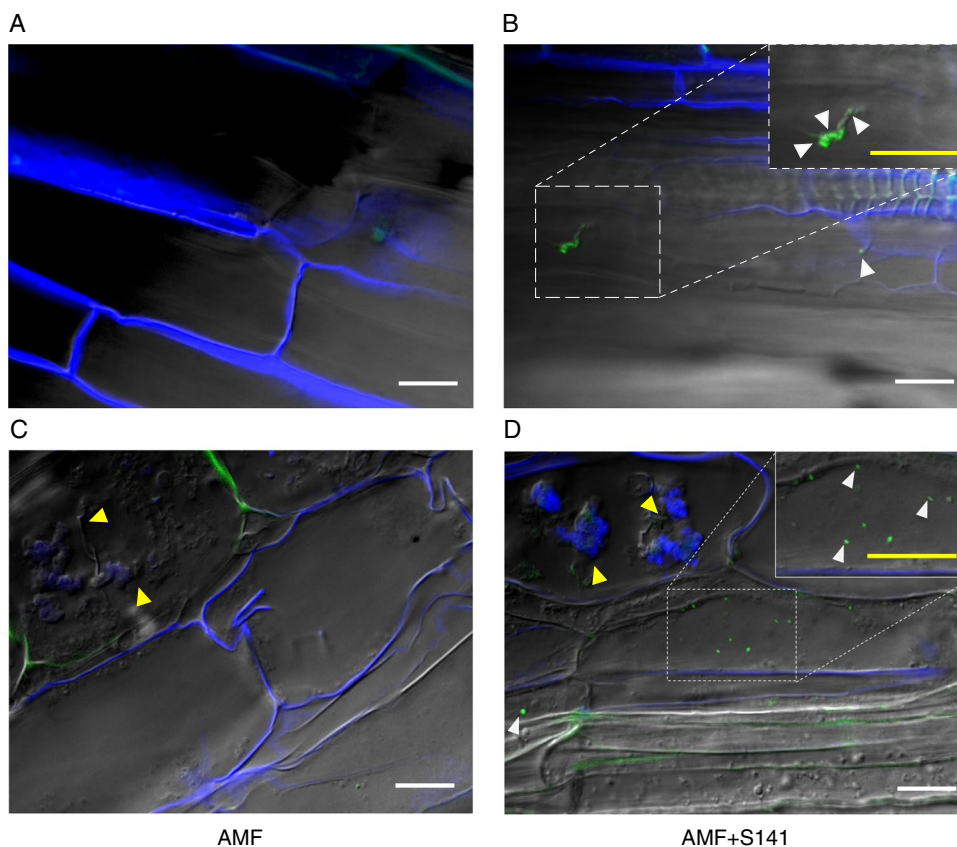
due to the co-inoculation could lead to enhancement in biomass and N and P concentrations of *L. japonicus*. Alternatively, the synergistic effects of *B. velezensis* S141 and AMF in the roots might have altered the gene expression patterns in both the plant and AMF, resulting in activation of plant and AMF growth.

Auxin is involved in the initiation of arbuscular mycorrhizal symbiosis and the development of arbuscules (Ho-Plágaro & García-Garrido 2022). *B. velezensis* S141 produces auxins, and disruption of the *IpyAD*, *yhcX*, and *dhaS* genes reduces auxin production to 4% to 30% of the wild-type strain (Sibponkrung et al. 2020). Using these mutant strains, we tested the potential role of auxins from *B. velezensis* S141 with respect to producing an increase in AMF abundance, but AMF colonization was similar to wild-type inoculation (Fig. 4). Such levels of auxin reduction might not affect AMF abundance, possibly due to compensatory effects from other auxin-producing genes. Another explanation for this finding could be that the auxin-producing capability of *B. velezensis* S141 is not a primary mechanism for promoting plant growth or AMF colonization. Nevertheless, *B. velezensis* S141 might employ other mechanisms that facilitate AMF–plant symbiosis, and these mechanisms should be further investigated.

B. velezensis S141 enhanced mycorrhizal formation in *L. japonicus*, a finding that is consistent with the activation of plant marker genes (*SbtM1* and *RAM1*) associated with mycorrhizal symbiosis (Fig. S2A). Similarly, the expression of the *HA1*, *PT4*, and *AMT2:1* genes, all of which are essential for nutrient uptake during mycorrhizal symbiosis, was also upregulated by inoculation with *B. velezensis* S141. This finding mirrors the observed increase in shoot N and P concentrations. These results suggest that the presence of endophytic *B. velezensis* S141 amplifies the benefits of mycorrhizal symbiosis. In contrast, the expression of genes involved in lipid transfer from plants to AMF (except for *RAM2*) remained unchanged after *B. velezensis* S141 inoculation. The different gene expression responses related to carbon supply and symbiotic nutrient uptake when exposed to *B. velezensis* S141 might influence the cost–benefit balance in mycorrhizal symbiosis.

Expression of AMF cell cycle-related genes is related to AMF proliferation in roots in response to environmental changes (Sugimura and Saito 2017). AMF cell cycle-related genes were found to be downregulated during the suppression of AMF colonization in roots caused by high phosphate exposure. In our experiments, the presence of *B. velezensis* S141 led to an increase in the expression of cell cycle-related genes in *R. irregularis* (Fig. S2B). This finding is consistent with the increase in abundance of *R. irregularis* in *L. japonicus* roots after inoculation with *B. velezensis* S141, thus supporting the occurrence of activation of AMF proliferation by the PGPR strain in terms of gene expression.

We also analyzed gene expression related to P metabolism, an important function in AM symbiosis, as an effect of the coexistence of *B. velezensis* S141 on AMF. Polyphosphate synthesized in AMF is an important source of P for plants, and the amount of polyphosphate in AMF is thought to be regulated through a metabolic balance between polyphosphate synthesis by the vacuolar transporter chaperone (VTC) complex and degradation by endopolyphosphatases (PPNs) (Ezawa and Saito 2018). Based on our gene expression analysis, the amount of PPN transcripts in AMF was not affected by *B. velezensis* S141 inoculation, but VTC transcripts had decreased (Fig. S2B). Although polyphosphate levels were not determined, polyphosphate may progress in the direction of degradation in intraradical hyphae and arbuscules of AMF. Since short-chain polyphosphate acts as a pool of P supply to the plant (Takanishi et al. 2009), the higher shoot P content of plants inoculated with *B. velezensis* S141 may be related to modulation of polyphosphate metabolism genes in AMF. In addition, the expression of *MST2*, which is involved in sugar transport to AMF cells, was downregulated after co-inoculation with *B. velezensis* S141.

Intriguingly, *B. velezensis* S141 was localized within plant cells, with no bacteria detected in the intercellular space (Fig. 5). This observation suggests a specific and targeted mode of communication, with the bacteria potentially relying on obtaining nutrients from living plant cells, indicative of a mutualistic relationship. These results shed light on the intricate mechanisms involved in nutrient and sugar acquisition, as well as delivery within the plant-AMF partnership. They also underscore the potential role of co-inoculation with an endophytic PGPR in these processes, warranting further investigation.

5 Conclusions

To summarize, our study sheds light on the beneficial effects of *B. velezensis* S141 on the plant-AMF symbiosis. The results suggest that *B. velezensis* S141 leads to enhancement of root growth in the early stages and later promotes the abundance of AMF. However, the production of a plant growth hormone, IAA, is not the key mechanism used by *B. velezensis* S141 to promote mycorrhizal symbiosis. Surprisingly, strain *B. velezensis* S141 was identified as an endophyte in *L. japonicus*, and this strain had the capability of inducing the expression of plant marker genes mainly involved in mycorrhizal formation and nutrient uptake. Moreover, induction of the expression of marker genes related to the cell cycle of *R. irregularis* when co-inoculated with *B. velezensis* S141 strongly supports its potential for promoting fungal growth. These results highlight the importance of exploring the potential of beneficial microbes in

enhancing AMF growth and promoting plant growth through the nutrient uptake pathway.

Declarations

Ethical approval The biosecurity concerns of this study were reviewed and approved by Suranaree University of Technology (approval number: SUT-IBC-05/ 2021) and are in accordance with the levels of risk in pathogens and animal toxins listed in "the Risk Group of Pathogen and Animal Toxin (2017)" issued by the Department of Medical Sciences, Ministry of Public Health, the Pathogen and Animal Toxin Act (2015) and Biosafety Guidelines for Modern Biotechnology, BIOTEC (2016).

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