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Co-Inoculation of Endophytic *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2: Growth Promotion and Plant–Microbe Interactions Across Diverse Crops

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Abstract

Plant growth-promoting bacteria can improve plant performance through interactions with host plants, although their effects across diverse hosts remain incompletely understood. This study investigated individual and combined inoculation with *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2 in eight plant species. Both strains were recovered from surface-sterilized roots, stems, and leaves of selected hosts, while confocal microscopy revealed fluorescence signals associated with plant tissues, consistent with bacterial colonization. Plant growth responses varied among hosts and inoculation treatments. Bacterial gene expression analysis revealed changes in transcripts associated with tryptophan metabolism, motility, cell envelope and exopolysaccharide-related functions, secretion, nitrogen-related processes, and ACC deaminase. Plant genes related to signaling, growth regulation, nutrient transport, and defense also showed differential expression. In a separate experiment using semi-solid plant growth medium, dual inoculation was associated with host-dependent changes in ascorbate peroxidase, catalase, and superoxide dismutase activities. Notably, reduced expression of selected SUTN9-2 genes in green chiretta (*Andrographis paniculata*) did not correspond to reduced plant growth responses. Overall, the findings highlight host-dependent responses to individual and combined bacterial inoculation and support further investigation of these strains as potential microbial inoculants for diverse crops.

Keywords: endophyte; *Bacillus velezensis*; *Bradyrhizobium*; potting soil; colonization; co-inoculation

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1. Introduction

The development of sustainable agricultural systems requires strategies that main-

tain crop productivity while reducing dependence on synthetic fertilizers and other chemical inputs. Beneficial plant-associated microorganisms have attracted considerable attention as biological inputs because of their capacity to improve plant nutrition, stimulate plant development, and enhance plant resilience to environmental stresses [1]. Among these microorganisms, plant growth-promoting rhizobacteria (PGPR) can promote plant growth through diverse direct and indirect mechanisms, including nutrient mobilization, phytohormone production, biological nitrogen fixation, siderophore production, modulation of root development, and induction of plant defense responses [2–4]. However, the effectiveness of PGPR is influenced by the compatibility among bacterial strains, host plants, and the surrounding soil environment, resulting in variable plant responses following microbial inoculation [5,6].

In addition to single-strain inoculation, combinations of beneficial microorganisms have increasingly been investigated because complementary microbial functions may enhance plant performance [7]. Co-inoculation of non-rhizobial PGPR with rhizobia has been reported to improve plant growth, nutrient acquisition, nodulation, and biological nitrogen fixation, particularly in leguminous crops [5,6]. Such interactions may involve complementary functions, including phytohormone production, nutrient mobilization, modification of the rhizosphere environment, and stimulation of rhizobial activity [6,8]. Thus, these non-rhizobial PGPR may function as helper microorganisms that act together with rhizobia through coordinated interactions rather than solely as independent plant growth promoters.

Bradyrhizobium is traditionally recognized for its ability to establish symbiotic associations with legumes and contribute to biological nitrogen fixation. However, the beneficial effects of rhizobia may extend beyond nodule formation and nitrogen fixation, as rhizobial bacteria can also interact with plant roots through phytohormones, nutrient acquisition, signaling molecules, and other plant growth-promoting traits [9]. Consequently, co-inoculation with *Bradyrhizobium* and non-rhizobial PGPR may provide complementary functions that influence plant development. Previous studies of non-rhizobial PGPR–*Bradyrhizobium* co-inoculation have predominantly focused on leguminous crops, particularly soybean, with an emphasis on nodulation and nitrogen fixation. Consequently, the effects of defined non-rhizobial PGPR–*Bradyrhizobium* combinations on a broader range of vegetable crops remain less well understood [5,6,10].

Among non-rhizobial PGPR, *Bacillus* spp. are particularly attractive for microbial inoculant preparation because of their diverse plant-beneficial functions and ability to form stress-resistant endospores [11]. *Bacillus* spp. can contribute to plant growth through phytohormone production, nutrient mobilization, phosphate solubilization, and suppression of plant pathogens, while their spore-forming capacity facilitates formulation, storage, and application under variable environmental conditions [12]. Moreover, *Bacillus* spp. can establish associations with plant roots and interact with other beneficial microorganisms. For example, *Bacillus velezensis* SQR9 and *Pseudomonas stutzeri* XL272 have been shown to co-colonize plant roots and interact through metabolic and biofilm-associated mechanisms, resulting in improved plant growth and salt-stress tolerance [13] . Such findings support the potential of *Bacillus*-based microbial consortia to provide complementary functions beyond those provided by individual bacterial inoculants.

Despite increasing interest in beneficial bacterial co-inoculation, the effects of combining benefical *Bacillus* with *Bradyrhizobium* on different plant hosts remain insufficiently understood [6]. In particular, it is unclear how co-inoculation with a defined combination of *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2 influences bacterial and plant responses across diverse vegetable crops grown in a common soil substrate. Furthermore, plant growth responses alone do not fully explain the biological basis of

microbial-mediated growth promotion. Bacterial establishment within plant tissues, changes in plant physiological responses, and transcriptional responses of both the plant host and bacterial partners may provide complementary evidence for understanding these interactions. Moreover, evaluating microbial inoculants under pot-culture conditions can provide a practical intermediate system between laboratory assays and field applications [6]. Such systems may also be relevant to small-scale or space-limited crop production, where appropriate growing substrates and effective microbial inoculation may contribute to plant productivity.

Endophytic bacteria are particularly promising as microbial inoculants because their ability to colonize internal plant tissues can facilitate close interactions with host plants. Through these associations, endophytes may influence plant growth, nutrient acquisition, hormone signaling, and stress responses, providing potential advantages over bacteria restricted primarily to the rhizosphere [14,15]. Among beneficial plant-associated bacteria, *Bacillus velezensis* S141 (S141), a non-rhizobial plant growth-promoting bacterium, and *Bradyrhizobium* sp. SUTN9-2 (SUTN9-2), a rhizobial bacterium, possess distinct but potentially complementary plant-associated traits. S141 has previously been shown to promote the growth of several crops, including tomato, chili, and melon [16,17]. In contrast, SUTN9-2 has been reported to establish endophytic associations with both leguminous and non-leguminous plants, including bean, chili, tomato, and rice [16,18]. Although *Bradyrhizobium* species are primarily known for their symbiotic associations with leguminous plants, their ability to establish associations with non-leguminous hosts suggests that their interactions with plants may extend beyond the conventional legume–rhizobium symbiosis. In non-leguminous plants, such interactions may involve endophytic or other plant-associated effects rather than nodule formation. Accordingly, the present study included eight plant species, comprising one legume (yardlong bean) and seven non-legumes, to investigate bacterial association with plant tissues and host-dependent plant responses following individual and dual inoculation. The inclusion of yardlong bean also allowed nodulation responses to be examined in a leguminous host, although the study was not designed to determine nitrogen fixation efficiency or nitrogen transfer to the plant. These findings may help clarify how the two bacterial strains respond to individual and combined inoculation and how their interactions with different plant hosts are reflected in bacterial gene expression and plant molecular responses. However, the effects of their combined inoculation on these responses across different host species remain insufficiently understood.

In the present study, we investigated the effects of single and co-inoculation with endophytic S141 and SUTN9-2 on the growth and physiological responses of diverse vegetable and medicinal plants cultivated in university-developed potting soil. Eight plant species representing different crop types were evaluated: yardlong bean (*Vigna unguiculata*), lettuce (*Lactuca sativa*), kale (*Brassica oleracea* var. *alboglabra*), Chinese kale (*Brassica rapa* subsp. *chinensis*), eggplant (*Solanum melongena*), tomato (*S. lycopersicum* var. *cerasiforme*), chili (*Capsicum annuum*), and green chiretta (*Andrographis paniculata*). Plants were evaluated under single- and co-inoculation conditions, with non-inoculated university and commercial potting soils included as reference treatments. Plant performance was assessed based on plant phenotype, chlorophyll content (SPAD), shoot and root growth, and fresh and dry biomass. Representative plant species showing pronounced responses to bacterial inoculation were subsequently selected for assessment of bacterial establishment within plant tissues using confocal laser scanning microscopy and culture-based bacterial enumeration. To further investigate plant responses, antioxidant enzyme activities and the expression of selected plant-responsive genes were examined. In parallel, the expression of selected bacterial genes was analyzed to evaluate bacterial re-

sponses during plant association. We hypothesized that co-inoculation of S141 and SUTN9-2 would provide complementary plant growth-promoting effects across diverse plant species and that these effects would be associated with coordinated physiological and transcriptional responses of both the plant host and bacterial partners.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Two bacterial strains with plant growth-promoting properties were used as inoculants in this study. The bacterial strains were obtained from the Applied Soil Microbiology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. S141 was routinely maintained on nutrient agar (NA) and cultivated in nutrient broth (NB) for preparation of bacterial inocula. SUTN9-2 was maintained on yeast-mannitol agar (YMA) and cultivated in yeast-mannitol broth prior to plant inoculation.

For bacterial colonization and localization analyses using confocal laser scanning microscopy (CLSM (Nikon Model Ni-E, Nikon Instech Co., Ltd., Tokyo, Japan)), fluorescently labeled derivatives of the two bacterial strains were used. S141 expressing green fluorescent protein (S141-GFP) [10] and SUTN9-2 expressing red fluorescent protein (SUTN9-2-DsRed) [18] were used to visualize bacterial localization within plant tissues. S141-GFP was maintained on NA supplemented with 2.5 μg/mL phleomycin, whereas SUTN9-2-DsRed was maintained on YMA supplemented with 100 μg/mL spectinomycin. The fluorescently labeled strains were cultivated under the same conditions as their corresponding parental strains prior to plant inoculation.

2.2. Pot Experiment and Experimental Design

Pot experiments were conducted to evaluate the effects of single and co-inoculation with S141 and SUTN9-2 on the growth and physiological responses of eight vegetable and medicinal plant species. The plant species included yardlong bean, lettuce, kale, Chinese kale, eggplant, tomato, chili, and green chiretta. Seeds were germinated and seedlings were grown in seedling trays containing a peat moss and coco coir mixture at a ratio of 3:1 (*v*/*v*) for 14 days before transplantation.

Fourteen-day-old seedlings were initially grown in seedling trays containing a 3:1 (*v*/*v*) mixture of peat moss and finely ground coconut coir. Uniform seedlings with a shoot length of approximately 5 cm were selected and individually transplanted into 8-inch pots containing either SUT potting soil (commercialized by the Suranaree University of Technology) or commercial potting soil. Commercial potting soil was included only as a non-inoculated reference for contextual comparison of plant growth in a commercially available growing substrate. Bacterial inoculation treatments were evaluated exclusively using the SUT growing substrate. Five biological replicates were used for each treatment. The SUT-developed potting soil consisted of raw rice husk, burned rice husk, filter-press cake, cassava peel residues, and topsoil at a ratio of 4:2:3:3:1, respectively, with a pH of 6.60 ± 0.14 and an electrical conductivity (EC) of 1.05 ± 0.19 dS/m.

The SUT potting soil was used as the common substrate for the bacterial inoculation treatments: non-inoculated control, S141, SUTN9-2, and co-inoculation with S141 and SUTN9-2. For single-strain inoculations, 10 mL of either S141 or SUTN9-2 suspension (10^8 CFU/mL) was added per kilogram of soil. For co-inoculation, the two bacterial suspensions were mixed at a 1:1 ratio (5 mL each) to maintain a total inoculum volume of 10 mL per kilogram of soil. Following bacterial inoculation, the SUT potting soil had a pH of 6.70 ± 0.14 and an EC of 1.07 ± 0.18 dS/m. Commercial potting soil was included only

as a non-inoculated reference treatment and was not used for bacterial inoculation. Bacterial inoculation was performed according to the designated treatment immediately after transplantation. The plants were cultivated under outdoor conditions at the organic farm of SUT (Nakhon Ratchasima, Thailand) during May–June 2025. Throughout the cultivation period, the potted plants were irrigated twice daily, in the morning and evening, with equal volumes of tap water per pot, adjusted as appropriate to maintain optimal soil moisture.

Plant growth and physiological parameters were monitored during cultivation. Plant phenotypes were recorded at 10, 14, 20, 25, and 30 days after inoculation (DAI). Leaf chlorophyll content was indirectly estimated using a SPAD meter at the same time points. For each plant, three readings were taken from the middle region of the third fully expanded leaf from the shoot apex, avoiding the midrib, and averaged to obtain one SPAD value per plant. At the final harvest at 30 DAI, plant height, root length, and fresh and dry biomass were determined (see the pot experimental design in Figure 1). For yardlong bean, nodulation was additionally assessed by determining nodule number and nodule dry weight following bacterial inoculation. Representative photographs of the nodules were also recorded.

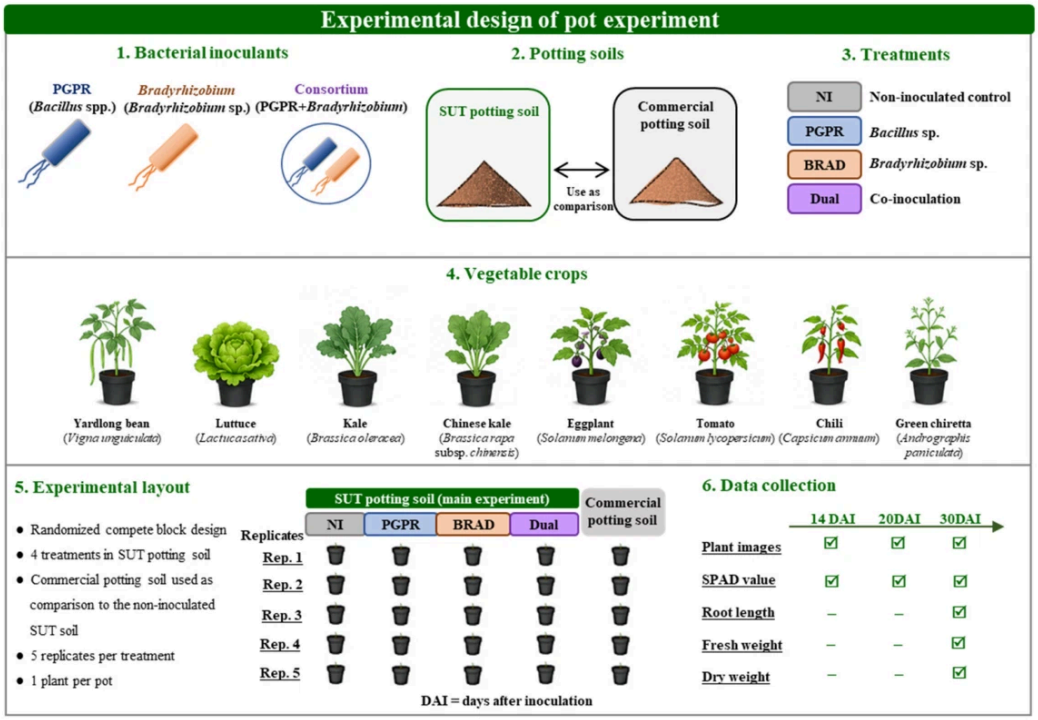


Figure 1. Experimental design of pot experiment. Illustrative images of the eight potted plant species were generated using ChatGPT (GPT-4o, OpenAI) from a single prompt: "Generate a single scientific illustration of eight individual potted plants arranged from left to right in this order: yardlong bean, lettuce, kale, Chinese kale, eggplant, tomato, chili, and green chiretta. Depict each species with realistic, characteristic growth habits and foliage. Use uniformly sized pots, small plants, generous and even spacing, and a plain white background. Do not include labels, text, or decorative elements." The AI-generated images are used for illustrative purposes only and do not represent experimental data. The overall experimental design and figure layout were prepared by the authors.

To further evaluate the magnitude of plant growth responses to bacterial inoculation, percentage changes in dry biomass, root length, and SPAD of plant values at 30 DAI were calculated for each treatment relative to the corresponding non-inoculated

control for each plant species, following the response-ratio approach described by Hedges et al. [19]. Positive and negative values indicate increases and decreases, respectively, relative to the control. The percentage changes are presented as descriptive measures in Supplementary Table S1, while statistical comparisons among treatments were performed separately using the original data and Tukey's honestly significant difference (HSD) test ($p < 0.05$).

2.3. In Vitro Assessment of Bacterial Colonization and Endophytic Establishment

Selected plant species showing pronounced responses to bacterial inoculation in the pot experiment were subsequently used for in vitro assessment of bacterial colonization and endophytic establishment. Eggplant, lettuce, kale, Chinese kale, green chiretta, and yardlong bean were selected as representative plant species. Seeds were surface-sterilized using a previously described procedure [20] and germinated on water semi-solid agar (0.8% agar) for approximately 3 days. The effectiveness of surface sterilization was confirmed by plating aliquots of the final rinse water onto NA plates. No bacterial colonies were observed after incubation, confirming the absence of culturable bacteria in the final rinse water [21]. Seedlings with newly emerged roots of approximately 0.3–0.5 mm in length were selected for bacterial inoculation. The seedlings were individually inoculated by immersion in suspensions of S141-GFP or SUTN9-2-DsRed, each adjusted to an optical density at 600 nm of 0.5, for 30 min. Following inoculation, the seedlings were transferred into semi-solid plant growth medium consisting of BNM medium supplemented with 0.1 M NH_4NO_3 and cultivated under controlled in vitro conditions with a 12 h light/12 h dark photoperiod.

Plant samples were collected at 7 and 14 DAI for analysis of bacterial localization and population density. For CLSM, samples collected at 7 DAI were sectioned into approximately 35–45 μm-thick sections using a vibratome and mounted on microscope slides for microscopic observation. S141-GFP and SUTN9-2-DsRed were visualized based on their respective green and red fluorescent signals to determine bacterial localization within plant tissues and evaluate potential endophytic establishment.

In parallel, bacterial populations associated with plant tissues were quantified at 7 and 14 DAI using a culture-dependent serial dilution plating method. Five representative plant species (tomato, kale, eggplant, lettuce, and Chinese kale) were selected from the eight plant species used in this study. Yardlong bean was excluded because nodule formation induced by *Bradyrhizobium* sp. SUTN9-2 could result in distinct bacterial populations, potentially complicating comparisons among host species, whereas Green chiretta was excluded because its small plant size would require a substantially larger number of plants to obtain sufficient tissue material for bacterial recovery analysis. Approximately 1 g of fresh plant tissue was collected and surface-sterilized to remove externally associated bacteria by treatment with 95% ethanol for 10 s, followed by three washes with sterile distilled water, treatment with 3% sodium hypochlorite for 5 min, and 10 subsequent washes with sterile distilled water. Sterilization efficiency was verified following the aforementioned final rinse water control procedure, which similarly showed no culturable bacteria. The surface-sterilized tissues were aseptically homogenized using a sterilized mortar and pestle with 1 mL of sterile saline (0.85% NaCl). Serial 10-fold dilutions were prepared and spread onto selective media. S141-GFP was enumerated on NA supplemented with 2.5 μg/mL phleomycin and incubated at 30 °C for 24 h, whereas SUTN9-2-DsRed was enumerated on YMA supplemented with 100 μg/mL spectinomycin and incubated at 30 °C for 5–7 days. Bacterial populations were expressed as colony-forming units per gram of fresh plant tissue (CFU/g fresh weight).

2.4. RNA Extraction and Purification

Plant species for gene expression analysis were selected based on the bacterial colonization results obtained from the in vitro experiments and the availability of genomic information for primer design. Eggplant was excluded from the gene expression analysis because suitable genomic information for target gene identification and primer design was not available in the NCBI database. Accordingly, green chiretta, tomato, and kale were selected for subsequent analysis; their reported genome sizes are approximately 284.3, 803.6, and 572 Mb, respectively, based on information available from the NCBI database.

The whole plant tissues were collected at 14 DAI from plants subjected to single inoculation with either bacterial strain, co-inoculation with both strains, and the non-inoculated control. Total RNA was extracted from the collected plant tissues using the FavorPrep Plant Total RNA Mini Kit (Favorgen Biotech Corp., Pingtung, Taiwan) according to the manufacturer's instructions. The extracted RNA was treated with DNase to remove genomic DNA contamination. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), whereas RNA integrity was evaluated using agarose gel electrophoresis. The absence of genomic DNA contamination was further confirmed by PCR using primers targeting the plant 18S rRNA gene (Table S2). RNA samples that passed the quality assessment were used for first-strand cDNA synthesis using the iScript Reverse Transcription Supermix (Bio-Rad). The synthesized cDNA was stored at −80 °C until quantitative real-time PCR (qRT-PCR) analysis.

2.5. Gene Expression Analysis of Plant and Bacterial Responses to Bacterial Inoculation

Relative expression levels of selected plant- and bacterial-responsive genes were determined by qRT-PCR using THUNDERBIRD Next SYBR qPCR Mix (TOYOBO) and gene-specific primers (Tables S1 and S2). For plant gene expression analysis, selected genes associated with plant growth and responses to bacterial inoculation were analyzed using actin as the internal reference gene. The target genes were selected to represent functional categories potentially involved in plant–microbe interactions, including signaling, growth regulation, nutrient transport, and defense responses. Specifically, the target genes were classified into three functional categories: plant signaling-related genes, including an ethylene-responsive transcription factor (ERF), a leucine-rich repeat (LRR) receptor-like serine/threonine kinase, and a jasmonate-induced protein (JIP); plant growth-related genes, including an auxin response factor (ARF), gibberellic acid insensitive (GAI), and a potassium transporter; and plant defense- and stress-response genes, including 1,3-glucanase, thaumatin-like protein, and pathogenesis-related (PR) protein (Table S2). These genes were selected to examine plant transcriptional responses potentially associated with growth regulation, signaling, nutrient acquisition, and defense following bacterial inoculation.

For bacterial gene expression analysis, target genes were selected based on their predicted or annotated functions in bacterial colonization, plant–bacterium interactions, cellular growth, nitrogen-related processes, ACC deaminase activity, and tryptophan metabolism potentially associated with IAA biosynthesis. The selected genes represent bacterial functions that may contribute to interactions with plant hosts, including nutrient-related metabolism, motility, cell envelope functions, secretion systems, and stress-related processes. The target genes analyzed in S141 were associated with tryptophan biosynthesis and transport (*trypC* and a tryptophan transport-related gene), bacterial motility and cell envelope functions (*flgG* and *tua*), and nitrogen-related cellular processes (*glnK* and *nifU*). In SUTN9-2, the target genes were associated with tryptophan biosynthesis (*trypC*), bacterial motility (*flgG*), exopolysaccharide biosynthesis (*exoB*), the

type III secretion system (*rhcN*), cell differentiation (*bclA*), nitrogen fixation-related processes (*nifH*), and ACC deaminase activity (*acdS*). The expression of these genes was assessed at the transcript level and was not intended to provide direct evidence of the corresponding physiological activities or establish causal relationships with plant growth responses.

Actin and 16S rRNA were used as reference genes for the normalization of plant and bacterial gene expression, respectively (Tables S1 and S2). The stability of 16S rRNA was evaluated by comparing its Ct values between single- and dual-inoculation treatments for each bacterial strain. Comparable Ct values were observed across treatments, supporting its use as a reference gene for bacterial gene expression analysis. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [22].

2.6. Determination of Antioxidant Enzyme Activities in Plants

Plant antioxidant enzyme activities were determined at 14 days after inoculation (DAI) using green chiretta, kale, and tomato plants grown on semi-solid plant growth medium. The experiment included two treatments, non-inoculated controls and plants co-inoculated with S141 and SUTN9-2, with three biological replicates per treatment. Whole-plant tissues, comprising roots, stems, and leaves, were collected at 14 DAI and immediately processed for crude enzyme extraction. The tissues were ground in liquid nitrogen and extracted using potassium phosphate buffer supplemented with polyvinylpyrrolidone (PVP) and ascorbic acid. The resulting supernatants were used to determine the activities of ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD).

APX activity was determined by monitoring the oxidation of ascorbate spectrophotometrically, following Souguir et al. [23], with minor modifications. The decrease in absorbance at 290 nm was recorded for 1 min at 25 °C. APX activity was calculated using the extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}), as described by Janda et al. [24].

CAT activity was determined by monitoring the decomposition of hydrogen peroxide (H_2O_2), following Fatnassi et al. [25] and Upadhyay et al. [26], with minor modifications. The decrease in absorbance at 240 nm was recorded for 1 min at 25 °C. CAT activity was calculated using the extinction coefficient of H_2O_2 (40 M^{-1} cm^{-1}).

SOD activity was determined using the nitroblue tetrazolium (NBT) photoreduction assay according to Fatnassi et al. [25], with minor modifications. The assay was based on the inhibition of light-induced NBT reduction by SOD. Absorbance was measured at 560 nm after 5 min of illumination. One unit of SOD activity was defined as the amount of enzyme required to inhibit NBT photoreduction by 50% under the assay conditions.

Protein concentrations in the crude enzyme extracts were determined using the Bradford method [27]. All enzyme activities were normalized to protein concentration and expressed as units per milligram of protein (U mg^{-1} protein). Detailed procedures for crude enzyme extraction and the determination of APX, CAT, and SOD activities are provided in the Supplementary Materials (Supplementary Figures S1 and S2).

2.7. Data Analysis and AI-Assisted Illustrations

Statistical analyses were performed using SPSS Statistics version 26.0 (IBM, Armonk, NY, USA). Data are presented as the mean ± standard deviation (SD). Treatment effects were analyzed separately for each plant species and experimental parameter using a general linear model (GLM) analysis of variance (ANOVA). When a significant treatment effect was detected, treatment means were compared using Tukey's honestly significant difference (HSD) test for post hoc pairwise comparisons. Differences were considered statistically significant at $p < 0.05$ [28,29]. Tukey's HSD was selected to

control the family-wise error rate associated with multiple comparisons. Formal diagnostic tests for normality and homogeneity of variance were not performed before ANOVA.

Generative AI was used (ChatGPT: GPT-4o, OpenAI) to generate illustrative elements for scientific figures. The authors incorporated these elements into the figures, reviewed the generated illustrations, and prepared the final figure layouts. The AI-generated elements are used for illustrative purposes only and do not represent experimental data. The corresponding figure legends provide details of the AI tool and prompts used.

3. Results

3.1. Assessment of SUT Soil Quality and Batch Consistency on Seed Germination

To evaluate the suitability of SUT potting soil inoculated with S141 and SUTN9-2 for subsequent plant cultivation, the germination and early growth of kale seeds were assessed. The biological effects of the bacterial mixed soil were compared against those of non-inoculated SUT soil and a water-only control. The results demonstrated high batch-to-batch consistency in soil quality, as reflected by stable kale germination and seedling development parameters across all three production rounds. These parameters included the number of root branches (plant^{-1}), total germinated seeds (out of 50), germination index (% GI), relative root growth (% RRG), and stem length (plant^{-1}) (Table 1 and Figure S1A–D).

Table 1. Effect of the bacterial mixed soil on seed germination and early seedling growth parameters of kale across three independent production batches.

	Control	SUT Soil	Mixed Soil
Batch 1.			
Branch of roots (plant^{-1})	2.76 ± 0.063 ^{c}	4.40 ± 0.39 ^{b}	5.10 ± 0.21 ^{a}
Total germinated seeds (50)	48.3 ± 1.15 ^{a}	48.66 ± 0.58 ^{a}	46.66 ± 1.53 ^{a}
% GI	100.03 ± 4.52 ^{c}	160.52 ± 13.74 ^{b}	178.47 ± 12.67 ^{a}
% RRG	100 ± 2.27 ^{c}	159.47 ± 14.27 ^{b}	184.69 ± 7.50 ^{a}
Stem length (plant^{-1})	2.87 ± 0.09 ^{c}	5.97 ± 0.47 ^{b}	6.53 ± 0.44 ^{a}
Batch 2.			
Branch of roots (plant^{-1})	3.47 ± 0.30 ^{c}	3.83 ± 0.33 ^{ab}	4.39 ± 0.30 ^{a}
Total germinated seeds (50)	19.67 ± 0.58 ^{a}	19.33 ± 0.58 ^{a}	19.33 ± 0.58 ^{a}
% GI	99.86 ± 6.31 ^{b}	108.6 ± 12.66 ^{b}	124.37 ± 7.54 ^{a}
% RRG	100 ± 8.62 ^{c}	110.29 ± 9.48 ^{b}	126.59 ± 8.59 ^{a}
Stem length (plant^{-1})	3.37 ± 0.09 ^{b}	6.06 ± 0.21 ^{a}	6.33 ± 0.15 ^{a}
Batch 3.			
Branch of roots (plant^{-1})	3.94 ± 0.61 ^{c}	4.74 ± 0.12 ^{b}	6.02 ± 0.37 ^{a}
Total germinated seeds (50)	20 ± 0.00 ^{a}	19.3 ± 0.58 ^{a}	20 ± 0.00 ^{a}
% GI	100 ± 15.49 ^{b}	116.17 ± 3.36 ^{b}	152.86 ± 9.47 ^{a}
% RRG	100 ± 15.49 ^{c}	120.2 ± 2.97 ^{b}	152.86 ± 9.47 ^{a}
Stem length (plant^{-1})	3.635 ± 0.61 ^{b}	8.06 ± 0.36 ^{a}	7.55 ± 0.29 ^{a}

Note: Values represent the mean ± standard deviation (SD) of three independent replicates. Distinct letters within the same column indicate statistically significant differences determined by Duncan's multiple range test ($p \leq 0.05$). % GI, Germination Index; % RRG, Relative Root Growth.

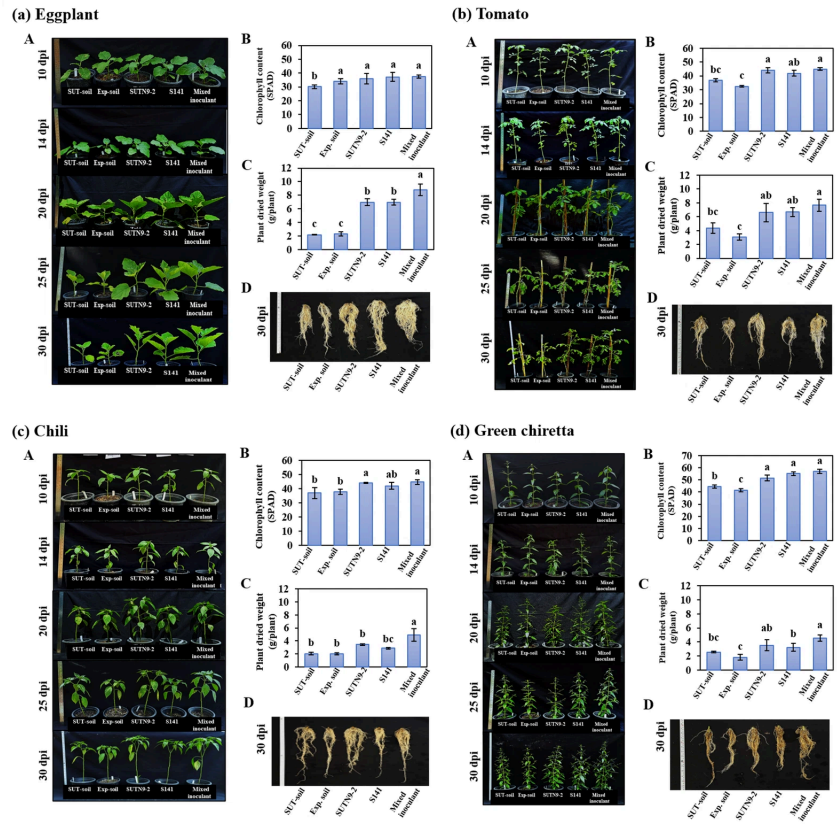
In all production rounds, the bacterial mixed soil significantly increased the number of root branches (plant^{-1}), % GI, and % RRG compared with both the non-inoculated SUT soil and water control. However, a slight fluctuation was noted in the second production batch regarding seedling stem length (plant^{-1}); unlike the other parameters, stem length in this particular batch did not show a statistically significant difference between bacterial mixed soil and the non-inoculated treatments (Table 1).

Taken together, these findings indicate consistent performance of the bacterial mixed soil across the three production batches, supporting its use in subsequent cultivation experiments.

3.2. Effects of Single and Co-Inoculation of Bacteria on Plant Growth Across Diverse Crops

The effects of single and co-inoculation with S141 and SUTN9-2 were evaluated in eight vegetable and medicinal plant species grown in SUT potting soil. SPAD were monitored at 10, 14, 20, 25, and 30 DAI, with non-inoculated SUT and commercial potting soils included as reference treatments (Figures 1 and S3).

Overall, bacterial inoculation improved plant growth compared with that in the non-inoculated controls. Differences among inoculation treatments became more apparent after 14 DAI, with co-inoculated plants generally showing more vigorous growth than single-inoculated plants, particularly in eggplant, tomato, chili, green chiretta, and yardlong bean (Figure 2). In contrast, lettuce, kale, and Chinese kale showed similar responses to single and co-inoculation, although both treatments generally differed from the non-inoculated controls. Changes in plant phenotype were accompanied by corresponding differences in SPAD values throughout the cultivation period, indicating improved chlorophyll status following bacterial inoculation (Figures 2 and S3).



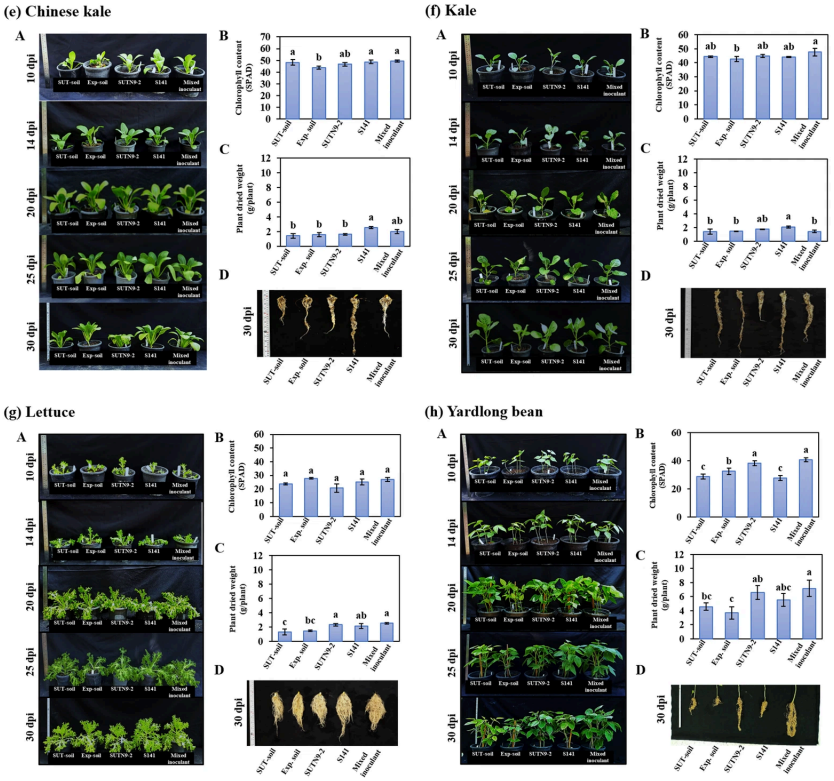


Figure 2. Effects of individual and dual bacterial inoculation on plant growth under pot-culture conditions. Representative phenotypes of plants inoculated with individual bacterial strains (*Bacillus velezensis* S141 or *Bradyrhizobium* sp. SUTN9-2) or their combination (S141 + SUTN9-2) were recorded at 10, 14, 20, 25, and 30 days after inoculation (DAI). The inoculation treatments were compared with two non-inoculated controls: university potting soil (SUT soil), and commercial potting soil (Exp. soil). At the final harvest (30 DAI), chlorophyll content (SPAD value), root length, and plant dry weight were determined. Plant species are indicated as follows: (1) eggplant, (2) tomato, (3) chili, (4) green chiretta, (5) Chinese kale, (6) kale, (7) lettuce, and (8) yardlong bean. Each treatment consisted of five biological replicates (one plant per pot). Data are presented as mean ± standard deviation (SD). Different uppercase letters indicate statistically significant differences among treatments within each plant species, as determined by Tukey's honestly significant difference (HSD) test ($p < 0.05$). Treatments sharing the same letter are not significantly different.

The percentage changes in dry biomass, root length, and SPAD values were calculated for each inoculation treatment relative to the corresponding non-inoculated control for each plant species (Supplementary Table S1). Among these parameters, percentage changes in dry biomass showed the clearest agreement with the observed plant phenotypes and dry biomass measurements. In six plant species, namely eggplant, tomato, chili, lettuce, green chiretta, and yardlong bean, the percentage increase in dry biomass followed the order of dual inoculation, SUTN9-2 alone, and S141 alone. In contrast, kale and Chinese kale showed greater increases in dry biomass following S141 inoculation than following dual inoculation or SUTN9-2 inoculation. These results indicate that plant growth responses varied among host species and bacterial inoculation treatments.

Nodulation was observed in yardlong bean under all treatments, including the non-inoculated soil controls. The highest nodule number was recorded in the dual-inoculation treatment, followed by SUTN9-2 alone, S141 alone, SUT soil, and experimental soil treatments (Figure S2). A similar trend was observed for nodule dry weight. However, the nodule dry weights under dual inoculation and SUTN9-2 alone were not significantly different. These results demonstrate differences in nodulation responses among

the treatments, although the contribution of nodulation to plant nitrogen acquisition was not evaluated in this study.

At the final assessment, inoculated plants generally exhibited greater fresh and dry biomass and root development than the non-inoculated controls, although the magnitude of the responses varied among plant species and inoculation treatments (Figure 2). The percentage changes in dry biomass further supported these observations, showing that dual inoculation generally produced the greatest increases in six plant species, namely eggplant, tomato, chili, lettuce, green chiretta, and yardlong bean, followed by SUTN9-2 and S141 inoculation alone. In contrast, S141 alone resulted in greater dry biomass increases in kale and Chinese kale than either SUTN9-2 alone or dual inoculation (Supplementary Table S1). These findings highlight the host-dependent nature of plant growth responses to bacterial inoculation, with the relative contributions of individual and combined inoculation varying among plant species.

3.3. Bacterial Colonization and Endophytic Establishment in Selected Plant Species

To investigate bacterial colonization and their association within plant tissues, selected plant species showing pronounced responses to bacterial inoculation were examined following single inoculation with S141-GFP or SUTN9-2-DsRed. Plant tissues (roots and stems) were collected at 7 DAI, surface sterilized, sectioned, and visualized using CLSM. Fluorescence signals from S141-GFP were observed in association with root hairs and epidermal regions, with additional signals detected in areas corresponding to the cortical region of the roots (Figure 3). Similar fluorescence patterns were observed across the selected host plants, indicating the association of S141 with plant root tissues. Likewise, fluorescence signals from SUTN9-2-DsRed were observed in association with root hairs and epidermal regions, as well as in areas corresponding to the root cortex (Figure 4). Although the orthogonal views or z-stack reconstructions are not available, these observations are consistent with bacterial populations within plant tissues of both bacterial strains.

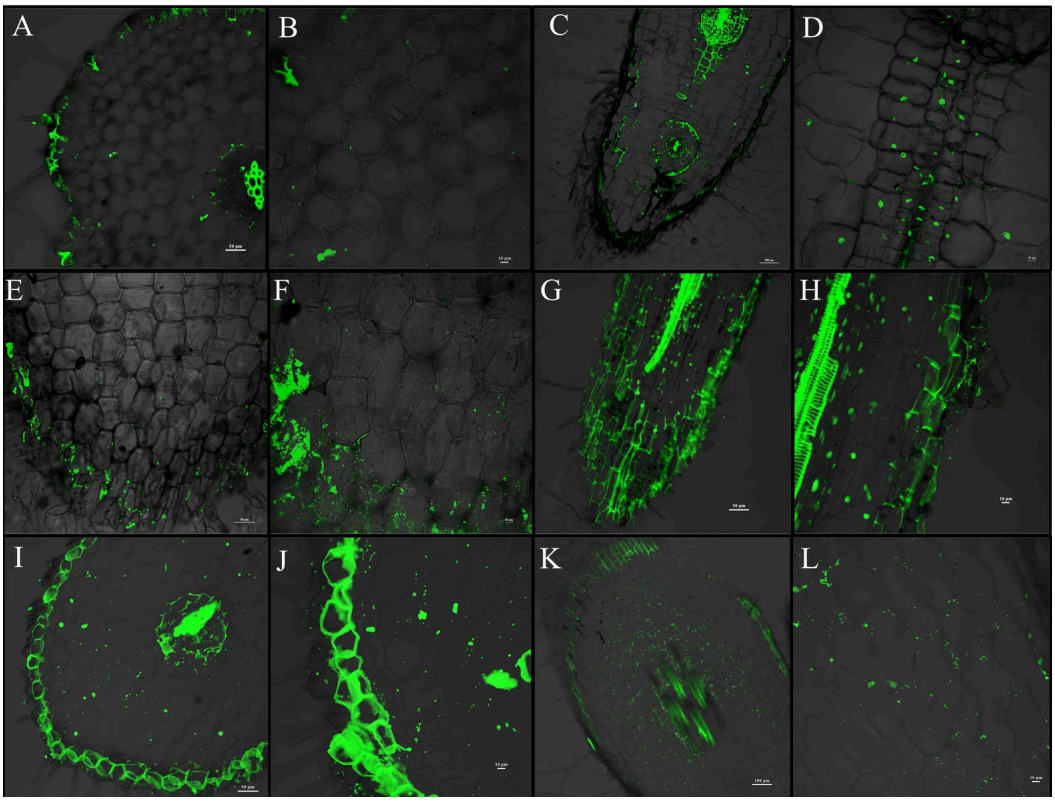


Figure 3. Confocal microscopic visualization of endophytic *Bacillus velezensis* S141 expressing green fluorescent protein (S141-GFP) in different host plant tissues. S141-GFP cells were visualized in plant tissues at 7 days after inoculation (DAI) using CLSM. Plant tissues were sectioned using a vibratome to a thickness of 30–45 μm before microscopic observation. From left to right, the image pairs show eggplant (**A**,**B**), kale (**C**,**D**), Chinese kale (**E**,**F**), lettuce (**G**,**H**), green chiretta (**I**,**J**), and yardlong bean (**K**,**L**). Low-magnification images (**A**,**C**,**E**,**G**,**I**,**K**) show overviews of the tissue sections, whereas the corresponding higher magnification images (**B**,**D**,**F**,**H**,**J**,**L**) show the same tissue regions in greater detail. Scale bars are indicated in each panel.

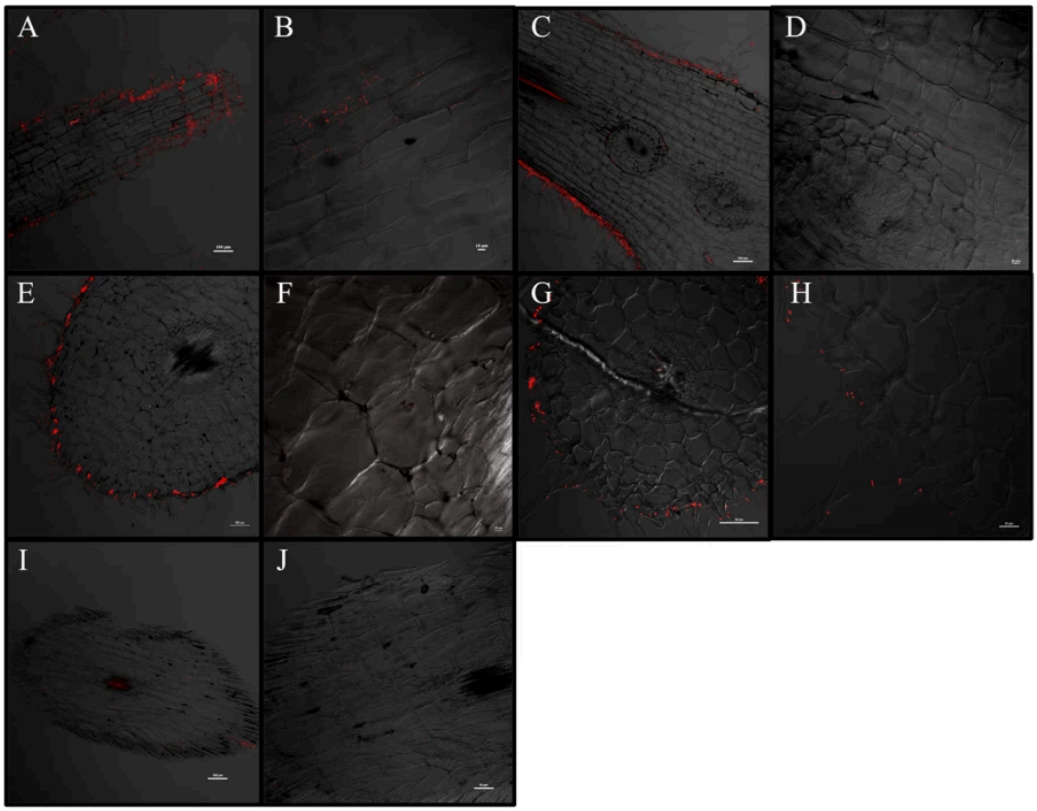


Figure 4. Confocal microscopic visualization of endophytic *Bradyrhizobium* sp. SUTN9-2-DsRed in different host plant tissues. SUTN9-2-DsRed cells were visualized in plant tissues at 7 days after inoculation (DAI) using CLSM. Plant tissues were sectioned using a vibratome to a thickness of 30–45 μm before microscopic observation. From left to right, the image pairs show eggplant (**A**,**B**), kale (**C**,**D**), Chinese kale (**E**,**F**), lettuce (**G**,**H**), and green chiretta (**I**,**J**). Low-magnification images (**A**,**C**,**E**, **G**,**I**) show overviews of the tissue sections, whereas the corresponding higher magnification images (**B**,**D**,**F**,**H**,**J**) show the same tissue regions in greater detail. Scale bars are indicated in each panel.

To quantitatively assess bacterial populations within plant tissues, S141-GFP and SUTN9-2-DsRed were recovered from surface-sterilized roots, stems, and leaves of tomato, kale, eggplant, lettuce, and Chinese kale at 7 and 14 DAI (Figure 5). S141-GFP was detected in all selected plant species, with populations of approximately 10^5 CFU/g fresh weight in tomato, lettuce, and Chinese kale and approximately 10^6 CFU/g fresh weight in kale and eggplant at both sampling times (Figure 5a–e). In comparison, SUTN9-2-DsRed was recovered from all five plant species at approximately 10^5 CFU/g fresh weight at both 7 and 14 DAI (Figure 5f–j). For both strains, bacterial populations were generally higher in root and stem tissues than in leaf tissues, indicating preferential colonization of belowground and basal plant tissues.

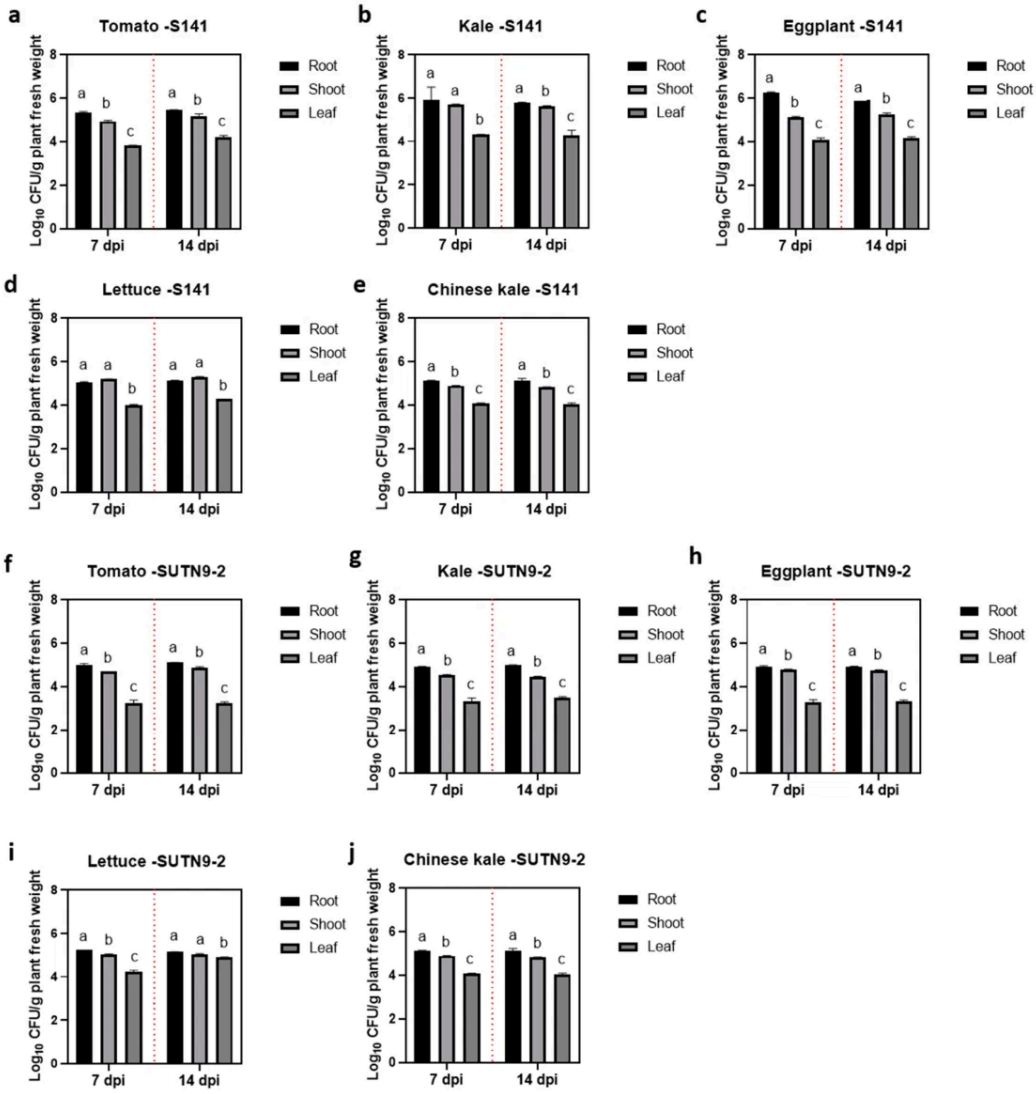


Figure 5. Culturable endophytic bacterial populations recovered from plant tissues following single inoculation with *Bacillus velezensis* S141 or *Bradyrhizobium* sp. SUTN9-2. Root, stem, and leaf tissues were collected at 7 and 14 days after inoculation (DAI), surface-sterilized, and plated to quantify viable bacteria associated with internal plant tissues. (**a**–**e**) show the populations of *B. velezensis* S141 recovered from tomato, kale, eggplant, lettuce, and Chinese kale, respectively, whereas (**f**–**j**) show the populations of *Bradyrhizobium* sp. SUTN9-2 recovered from the same plant species in the same order. Bacterial populations are expressed as CFU/g fresh weight (FW). Data are presented as the mean ± standard deviation (SD) of three biological replicates ($n = 3$). Different letters indicate statistically significant differences among root, stem, and leaf tissues within each sampling time (7 or 14 DAI), as determined by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

Together, the CLSM observations and recovery of viable bacteria from surface-sterilized plant tissues provide complementary evidence that both S141 and SUTN9-2 can colonize internal plant tissues and establish an endophytic association with the selected host plants. These findings provide a biological basis for further investigating the physiological and molecular responses of plants and bacterial partners following inoculation.

3.4. Plant Transcriptional Responses to Bacterial Inoculation

The expression patterns of selected plant genes in three representative plant species, green chiretta, tomato, and kale, were analyzed using qRT-PCR (Figure 6). Gene expres-

sion was compared among plants inoculated with S141, SUTN9-2, or both strains (co-inoculation), relative to that in non-inoculated plants.

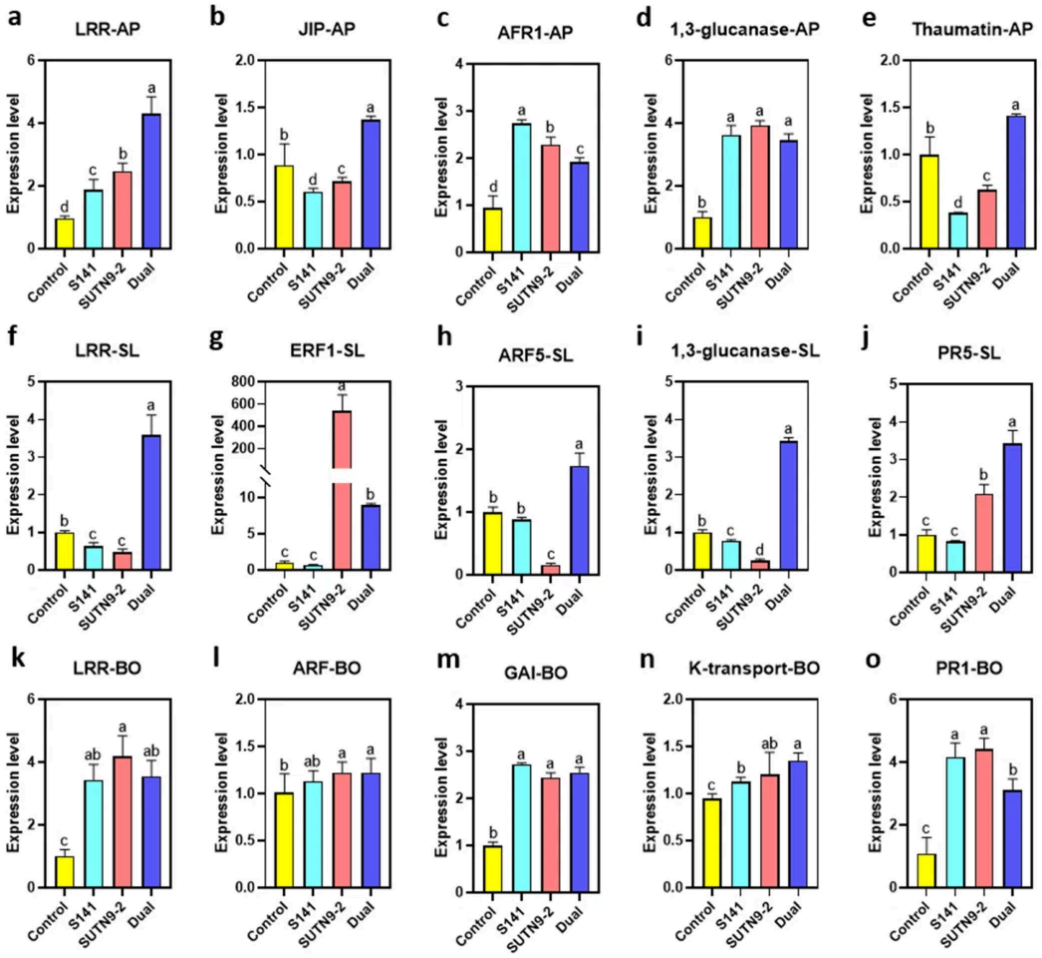


Figure 6. Relative expression of plant genes involved in signaling, growth, and defense-related pathways in response to bacterial inoculation. Gene expression was analyzed in green chiretta (*Andrographis paniculata*, AP), tomato (*Solanum lycopersicum* var. *cerasiforme*, SL), and kale (*Brassica oleracea* var. *alboglabra*, BO) plants at 14 days after inoculation (DAI) following inoculation with *Bacillus velezensis* S141, *Bradyrhizobium* sp. SUTN9-2, or both strains (Dual), with expression compared with that in non-inoculated plants (Control). Panels (**a**–**e**) show LRR, JIP, AFR1, 1,3-glucanase, and thaumatin, respectively, in green chiretta. Panels (**f**–**j**) show LRR, ERF1, ARF5, 1,3-glucanase, and PR5, respectively, in tomato. Panels (**k**–**o**) show LRR, AFR, GA1, K-transport, and PR1, respectively, in kale. Gene expression was quantified using qRT-PCR. Data are presented as the mean ± standard deviation (SD) of four biological replicates ($n = 4$). Different letters above the bars indicate statistically significant differences among treatments within each gene, as determined by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

In green chiretta (AP; *Andrographis paniculate*), several selected genes showed increased expression following bacterial inoculation. The expression of *LRR*, *ARF1*, and 1,3-glucanase was higher in both single- and co-inoculated plants than in the non-inoculated control (Figure 6a,c,d). Among these genes, *LRR* showed the highest expression in co-inoculated plants, exceeding the levels observed following single inoculation. In contrast, *ARF1* showed higher expression in plants inoculated either with S141 or SUTN9-2 than in co-inoculated plants. A distinct response pattern was observed for JIP and thaumatin-like protein: expression was higher following co-inoculation than in the control,

whereas both single-inoculation treatments resulted in lower expression than in the non-inoculated plants (Figure 6b). In tomato (SL; *Solanum lycopersicum* var. *cerasiforme*), co-inoculation generally resulted in the highest expression of several selected genes, including *LRR*, *ERF1*, *ARF5*, 1,3-glucanase, and *PR5*, compared with that in the other treatments (Figure 5f–j). In addition, *PR5* expression was higher in plants inoculated with SUTN9-2 than in those inoculated with S141 or the non-inoculated control. Notably, *ERF1* expression was markedly higher following SUTN9-2 inoculation than following the other treatments. In kale (BO; *Brassica oleracea* var. *alboglabra*), the expression patterns of the selected genes were generally more similar among the bacterial inoculation treatments than those observed in green chiretta and tomato. The expression levels of LRR, ARF, gibberellic acid insensitive, and the potassium transporter did not differ markedly between single- and co-inoculated plants (Figure 5k–n). In contrast, PR1 showed higher expression following single inoculation with either S141 or SUTN9-2 than in co-inoculated and non-inoculated plants, with the highest expression observed in the S141 treatment (Figure 6o).

Overall, bacterial inoculation altered the expression of genes associated with plant signaling, growth-related processes, and defense- and stress-response pathways, although the direction and magnitude of the responses varied among plant species and bacterial inoculation treatments. Notably, several genes, particularly JIP and thaumatin-like protein in green chiretta and LRR, ERF1, ARF5, 1,3-glucanase, and PR5 in tomato, showed distinct responses to co-inoculation compared with those to single inoculation.

3.5. Bacterial Transcriptional Responses to Plant Inoculation

The expression patterns of selected bacterial genes were investigated under single- and dual-inoculation conditions in three representative plant species—green chiretta, tomato, and kale—at 14 DAI. For S141, the selected target genes were detected at the transcript level in both single- and dual-inoculation treatments. Notably, dual inoculation was associated with higher expression levels of genes related to tryptophan biosynthesis and transport (*trypC* and the tryptophan transport-related gene), nitrogen-related cellular processes (*nifU* and *glnK*), and bacterial cell envelope and motility functions (*tua* and *flgG*). Across all three plant species, the expression levels of these genes were higher under dual inoculation than under the corresponding single-inoculation treatment (Figure 7A,C,E).

For SUTN9-2, the expression levels of selected genes associated with tryptophan biosynthesis (*trypC*), ACC deaminase production (*acdS*), cell differentiation (*bclA*), nitrogen fixation-related processes (*nifH*), the type III secretion system (*rhcN*), and exopolysaccharide biosynthesis (*exoB*) were examined. In tomato and kale, dual inoculation was generally associated with higher expression levels of all selected target genes than inoculation with SUTN9-2 alone. In contrast, green chiretta showed an opposite expression pattern, with lower expression levels of all selected SUTN9-2 genes under dual inoculation than under single inoculation (Figure 7B,D,F). These findings represent differences in bacterial gene expression at the transcript level under the tested inoculation conditions. In particular, the expression of *nifU*, *glnK*, and *nifH* does not by itself demonstrate nitrogen fixation activity or nitrogen transfer to the host plants.

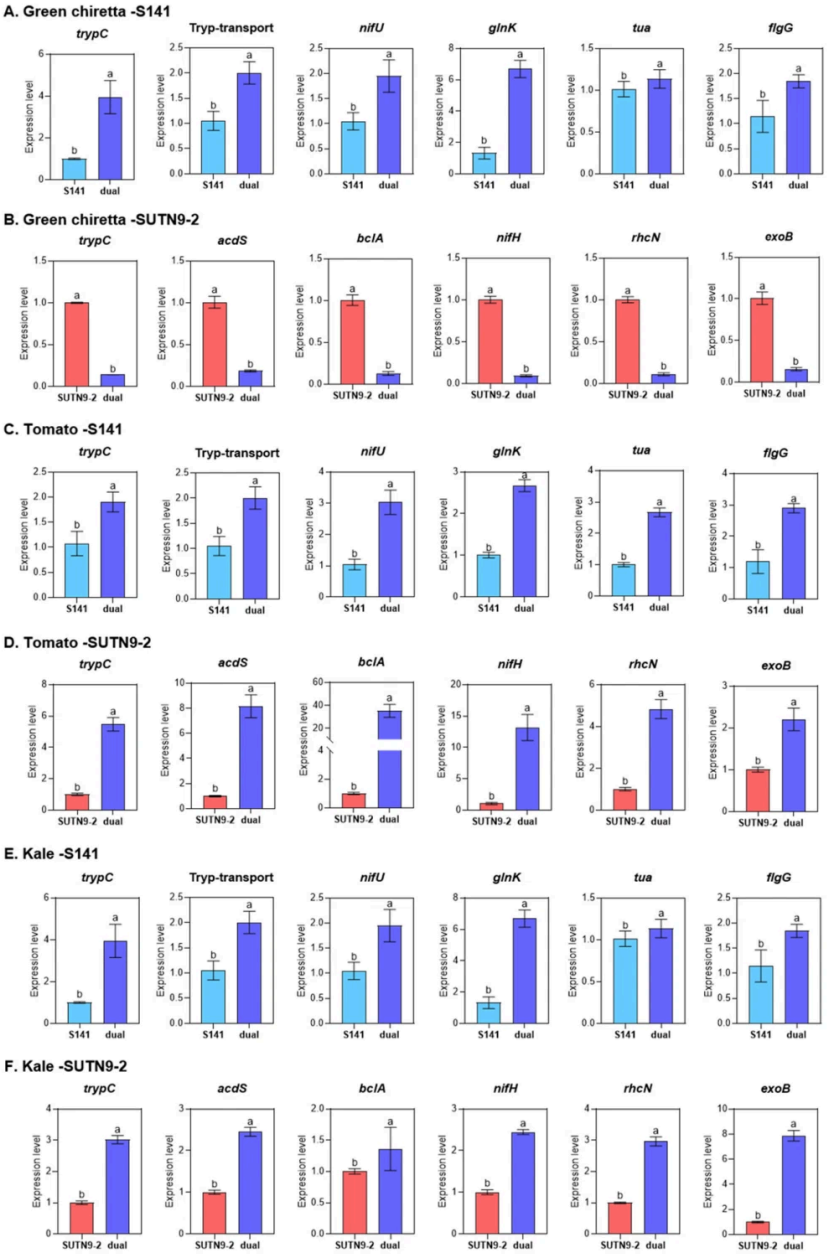


Figure 7. Relative expression of bacterial genes in plants following single- and dual-bacterial inoculation. The expression of selected genes in *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2 was analyzed in plants inoculated with either a single bacterial strain (S141 or SUTN9-2) or both strains simultaneously (Dual). For S141, the target genes included *trypC*, *tua*, *flgG*, *glnK*, *nifU*, and a tryptophan transport-related gene, representing genes associated with bacterial metabolism, motility, nitrogen metabolism, and plant–bacterium interactions. For SUTN9-2, the target genes included *exoB*, *flgG*, *rhcN*, *bclA*, *nifH*, and *acdS*, representing genes associated with exopolysaccharide biosynthesis, motility, secretion systems, nitrogen fixation, and plant growth promotion, respectively. Gene expression was quantified using qRT-PCR in bacterial cells associated with the inoculated plants. Data represent the mean ± standard deviation (SD) of four biological replicates ($n = 4$). Different letters above the bars indicate statistically significant differences among treatments according to Tukey's honestly significant difference (HSD) test ($p < 0.05$).

3.6. Co-Inoculation Modulates Antioxidant Enzyme Activities in Representative Plant Species

To further assess antioxidant responses associated with bacterial inoculation, the activities of three antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), and

superoxide dismutase (SOD), were determined in three representative plant species: green chiretta, kale, and tomato (Figure 8). Plants were grown on semi-solid plant growth medium, and tissues were collected at 14 days after inoculation (DAI) from non-inoculated controls and plants receiving dual inoculation with *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2. Compared with the corresponding non-inoculated controls, dual-inoculated green chiretta (Figure 8a–c) and tomato (Figure 8g–i) showed significantly higher activities of APX, CAT, and SOD. In kale, APX activity was significantly higher following dual inoculation, whereas CAT and SOD activities did not differ significantly between the two treatments (Figure 8d–f).

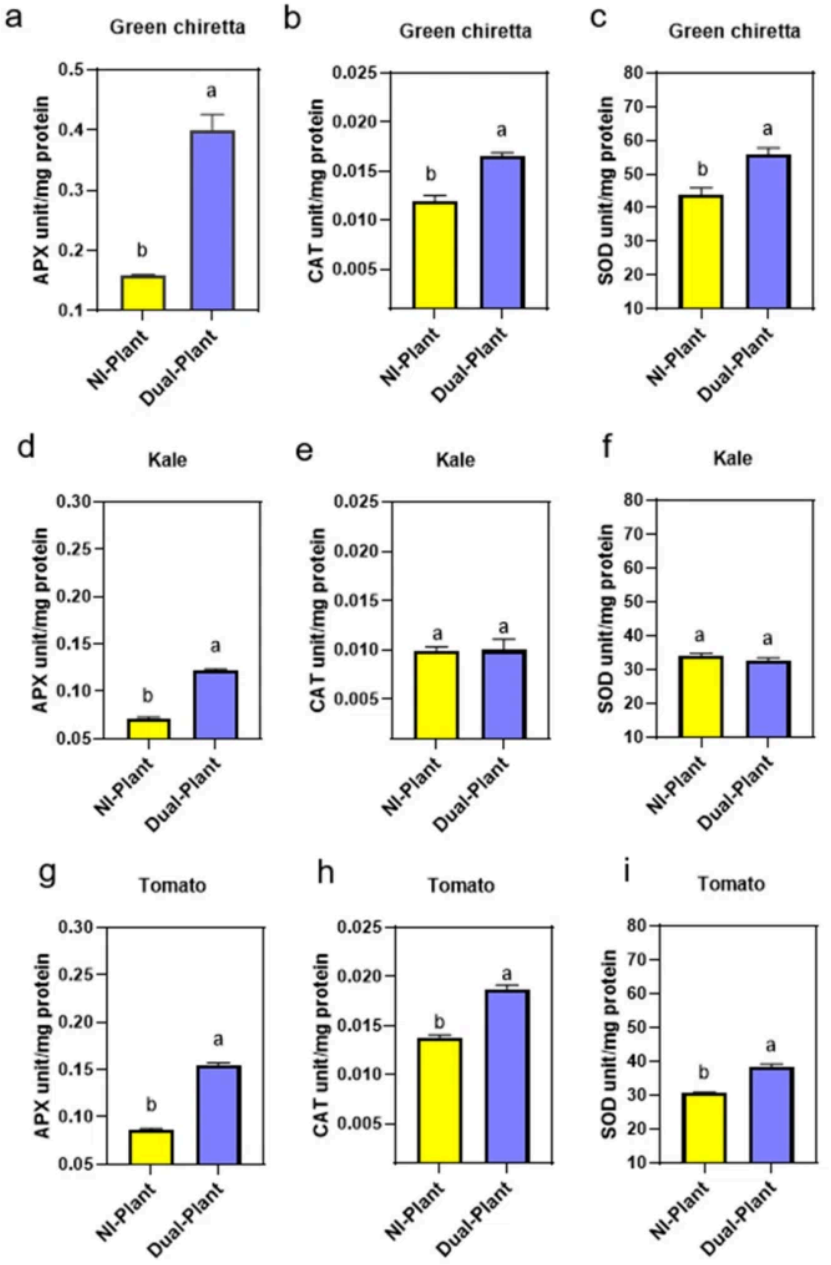


Figure 8. Effects of bacterial co-inoculation on antioxidant enzyme activities in representative vegetable crops. The activities of ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) were evaluated in green chiretta, kale, and tomato plants at 14 days after inoculation (DAI). Plants co-inoculated with *Bacillus velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2 (Dual) were compared with non-inoculated plants (NI) to assess changes in antioxidant defense responses associated with bacterial co-inoculation. (**a**–**c**), (**d**–**f**), and (**g**–**i**) show APX, CAT, and SOD

activities in green chiretta, kale, and tomato, respectively. Data are presented as the mean ± standard deviation (SD) of three biological replicates (n = 3). Different letters above the bars indicate statistically significant differences between treatments, as determined by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

Among the three enzymes, APX showed the largest relative increase, with activity approximately two-fold higher in kale and tomato and more than five-fold higher in green chiretta than in the respective non-inoculated controls (Figure 8a,d,g). Overall, the results indicate that dual inoculation was associated with changes in antioxidant enzyme activities under semi-solid plant growth conditions, with the magnitude and pattern of responses varying among plant species.

4. Discussion

The present study demonstrates that the bacterial inoculation system based on S141 and SUTN9-2 was associated with improved plant growth responses across a diverse range of vegetable and medicinal crops under the tested pot-culture conditions. The consistent germination and early seedling growth observed across three independently prepared substrate batches (Table 1 and Figure S1) indicate that the prepared SUT growing substrate had relatively consistent properties for supporting plant establishment. This consistency is relevant to the subsequent evaluation of S141 and SUTN9-2, as substrate properties can influence bacterial survival, establishment, and inoculant performance [30]. However, the three-batch germination trial was designed to assess substrate consistency and suitability for plant establishment, rather than to independently validate the reproducibility of bacterial inoculation effects across batches. Furthermore, germination and early seedling development provide functional indicators of substrate suitability but do not constitute a comprehensive assessment of inoculant performance [31]. Further characterization of physicochemical properties, bacterial viability, and population dynamics would be required to establish the batch-to-batch stability of the bacterial-inoculated substrate.

The present pot experiment was conducted using a single batch of the SUT growing substrate during one experimental season, with five biological replicates per treatment. Therefore, although the consistency of the prepared substrate was assessed separately using a three-batch germination trial, the biological effects of bacterial inoculation were not independently validated across multiple soil batches or growing seasons. This limitation should be considered when interpreting the findings, and further studies involving different soil batches, growing seasons, and field conditions are required to evaluate the consistency and broader applicability of the observed plant responses.

Both S141 and SUTN9-2 promoted plant growth across the selected plant species, although the magnitude of the response varied among hosts. The stronger responses observed at later stages are consistent with the time required for bacterial establishment and development of subsequent plant–microbe interactions (Figure 2). Root colonization by beneficial bacteria can be accompanied by changes in plant hormonal signaling, root development, and immune responses, as well as by bacterial production of growth-promoting metabolites [32]. *Bacillus velezensis* is particularly well characterized for its ability to colonize plant roots and promote plant growth through multiple mechanisms, including phytohormone production, nutrient acquisition, volatile compounds, and modulation of plant immunity [32,33].

The stronger responses observed with co-inoculation in several plants suggest that S141 and SUTN9-2 may provide partially complementary functions rather than acting through a single growth-promoting mechanism. Previous work with S141 and *Bradyrhizobium* has demonstrated that their combined application can enhance plant

growth and symbiotic performance, supporting the potential benefits of co-inoculation with PGPR and rhizobial partners [10,34]. A similar comparative approach was used by Padilha et al. [35], who evaluated individual and combined inoculation treatments in chickpea and assessed plant growth, nodulation, and nitrogen accumulation. Their findings illustrate the value of evaluating individual bacterial contributions alongside co-inoculation effects. In comparison, the present study examined host-dependent responses across eight plant species, but detailed nodulation and nitrogen-related measurements were available only for yardlong bean, the sole legume included in our study.

However, the absence of an additional response to co-inoculation in some hosts indicates that such effects are not universal. This host dependency is consistent with the role of plant genotype, root exudate composition, developmental stage, and the surrounding microbial environment in shaping bacterial recruitment and plant–microbe interactions [26]. The corresponding changes in SPAD values, biomass, and root development indicate that bacterial inoculation influenced multiple aspects of plant performance (Figures 2 and S3). Thus, the growth-promoting phenotype observed in the present study is likely to reflect the combined effects of bacterial colonization, nutrient-related functions, hormone-associated signaling, and modulation of plant physiological responses rather than a single bacterial trait.

The combined confocal microscopy observations and recovery of viable fluorescent bacteria from surface-sterilized tissues support the ability of both S141 and SUTN9-2 to colonize internal plant tissues across multiple hosts (Figures 3 and 4). The detection of bacterial fluorescence beyond the root surface, including the cortical region, is consistent with previous reports of endophytic or internal colonization by *B. velezensis* [33]. GFP-labeled *B. velezensis* strains have been visualized in root epidermal and cortical regions, while viable bacteria have also been recovered from surface-disinfected plant tissues, supporting their ability to persist within the plant interior [36,37]. Similarly, DsRed-tagged SUTN9-2 has been visualized within the epidermal and cortical regions of chili and tomato roots, providing direct evidence of its endophytic colonization of plant tissues [38].

The recovery of culturable bacteria from roots, stems, and leaves at both 7 and 14 DAI indicates that S141 and SUTN9-2 were associated within plant tissues during the early stages following inoculation (Figure 5). The greater recovery of bacteria from roots and stems than from leaves is consistent with the root serving as a major site of bacterial association, with bacterial occurrence in aerial tissues also observed in some plant–bacterium associations [39]. Furthermore, the agreement between CLSM observations and the recovery of culturable bacteria from surface-sterilized tissues provides complementary evidence of bacterial association within plant tissues. The absence of bacterial colonies from the final rinse water further supports the effectiveness of the surface-sterilization procedure. Nevertheless, these findings should be interpreted with consideration of the limitations of the methods, as bacteria residing in protected external niches cannot be completely excluded, and the available CLSM images do not provide definitive evidence of localization within specific tissue layers. In addition, because bacterial recovery was assessed only at 7 and 14 DAI, the results provide evidence of bacterial association at these two sampling points but do not establish long-term persistence or the dynamics of colonization over time.

Importantly, bacterial colonization or changes in the root-associated microbiome do not necessarily translate into measurable improvements in plant growth or yield. For example, de Andrade da Silva et al. [40] reported changes in the soybean root microbiome and increased colonization by *Bradyrhizobium japonicum* following bacterial inoculation, but no significant differences in grain yield among treatments. This finding highlights

the importance of distinguishing bacterial association from direct evidence of growth promotion.

The qRT-PCR results demonstrate that bacterial inoculation altered the expression of genes associated with plant signaling, growth regulation, and defense, with responses varying according to both host species and bacterial treatment [41,42]. The plant genes examined in this study were selected to represent different aspects of plant responses potentially involved in plant–microbe interactions, including signaling, growth regulation, nutrient transport, and defense responses. These gene-expression analyses were intended to provide complementary information on host transcriptional responses to bacterial inoculation rather than to establish direct functional relationships between individual genes and plant growth phenotypes.

In green chiretta, bacterial inoculation modulated genes associated with plant signaling and defense, as indicated by the induction of LRR, ARF1, and 1,3-glucanase (Figure 6a,b,d). The higher LRR expression under co-inoculation may reflect enhanced responsiveness to bacterial-derived signals, although the specific receptors or signaling molecules involved remain unclear. Co-inoculation also increased JIP and thaumatin-like protein expression (Figure 6b,e), suggesting a transcriptional response distinct from those observed with either single-inoculation treatment. In tomato, co-inoculation induced stronger expression of LRR, ERF1, ARF5, 1,3-glucanase, and PR5 (Figure 6f–j), indicating modulation of both growth- and defense-associated pathways. These responses are consistent with previous reports showing that plant growth-promoting bacteria can modify plant transcriptional networks involved in hormone signaling and defense responses [43,44].

In contrast, kale showed a more similar transcriptional response among bacterial treatments, with PR1 showing the strongest induction following S141 inoculation (Figure 6o). This variation further indicates that co-inoculation does not necessarily produce a stronger molecular response across all hosts. Instead, the outcome may depend on host-specific signaling networks and the interactions between the bacterial partners [42]. Importantly, the induction of defense-related genes should not automatically be interpreted as beneficial to plant growth or as evidence of enhanced disease resistance. The activation of defense responses may involve a growth–defense trade-off because the allocation of energy and resources to defense mechanisms may occur at the expense of plant growth, depending on the intensity and duration of the response. In the present study, the absence of a consistent relationship between defense-gene expression and plant growth suggests that the growth responses associated with S141 and SUTN9-2 cannot be explained solely by enhanced defense responses but may involve the integration of growth-, nutrient-, and defense-related processes.

An important feature of this study was the analysis of bacterial transcriptional responses alongside plant responses. The bacterial genes were selected based on their annotated or predicted functions potentially relevant to plant–bacterium interactions, including tryptophan metabolism, motility, cell-envelope and exopolysaccharide functions, secretion, nitrogen-related processes, and ACC deaminase activity. These analyses were intended to examine bacterial transcriptional responses under different host and inoculation conditions, rather than to directly demonstrate the corresponding physiological activities or their contributions to plant growth.

In S141, the higher expression of *trpC* and the tryptophan transport-related gene under co-inoculation is consistent with changes in tryptophan-associated metabolism (Figure 7), which may influence the availability of precursors for indole-related compound production. Tryptophan-dependent pathways are recognized contributors to bacterial IAA biosynthesis, and *B. velezensis* can produce IAA and other metabolites that in-

fluence plant development [10]. However, the expression of these genes alone does not confirm increased IAA production. The increased expression of *nifU* and *glnK* (Figure 7) further suggests modulation of nitrogen-related cellular processes, although their expression alone does not demonstrate increased nitrogen fixation or nitrogen transfer to the plant [45,46]. Similarly, the increased expression of *tua* and *flgG* (Figure 7) may reflect changes in bacterial cell-envelope-associated and motility-related functions, respectively, which could be relevant to bacterial persistence in plant-associated environments. Such functions are consistent with the established roles of cell-surface properties, motility, adhesion, and biofilm formation in *B. velezensis* root colonization [47–49].

A similar pattern was observed for SUTN9-2 in tomato and kale, where co-inoculation generally increased the expression of selected genes associated with tryptophan metabolism, ACC deaminase activity, exopolysaccharide production, nitrogen fixation-related processes, secretion, and cell differentiation (Figure 7D,F). The induction of *acdS* is particularly relevant because ACC deaminase-producing plant-associated bacteria can modulate plant ethylene levels and contribute to plant growth under conditions in which stress-associated ethylene would otherwise restrict growth [50,51]. Nevertheless, increased *acdS* transcript levels do not directly establish greater ACC deaminase activity or changes in plant ethylene levels. Similarly, increased *nifH* expression indicates a change in the transcription of a nitrogen fixation-related gene, but does not demonstrate increased nitrogenase activity or nitrogen transfer to the host plant. Direct measurements of enzyme activity and plant nitrogen acquisition would be necessary to establish these functional outcomes.

The interpretation of nitrogen-related gene expression should also be distinguished from the direct assessment of biological nitrogen fixation. Zveushe et al. [52], for example, evaluated nitrogen fixation in soybean using the percentage of nitrogen derived from the atmosphere (%Ndfa), together with measurements of nodule number and nodule dry weight. These measurements provide physiological and quantitative evidence of nitrogen fixation that cannot be inferred from transcript abundance alone. In the present study, although nitrogen-related genes were differentially expressed and nodulation was observed in yardlong bean, neither %Ndfa nor plant nitrogen accumulation was determined. Therefore, the observed transcriptional responses should be interpreted as changes in nitrogen-related gene expression rather than direct evidence of nitrogen fixation or nitrogen transfer to the host plant.

In contrast, all selected SUTN9-2 genes showed lower expression under co-inoculation in green chiretta than under single inoculation (Figure 7B). This host-specific response suggests that co-inoculation does not uniformly increase bacterial gene expression but may instead alter the physiological environment experienced by SUTN9-2 through host-derived signals, resource availability, or interactions with S141. Such host-dependent bacterial transcriptional responses are consistent with previous reports showing that plant root exudates can differentially regulate bacterial genes involved in metabolism and plant–microbe interactions [45,46]. Furthermore, the lower expression of the selected SUTN9-2 genes in green chiretta was not accompanied by a corresponding reduction in plant growth responses, indicating that the expression levels of the selected bacterial genes were not necessarily linearly associated with plant growth promotion under the conditions examined.

The increased activities of APX, CAT, and SOD in green chiretta and tomato, together with the selective increase in APX activity in kale (Figure 8), indicate that bacterial inoculation was associated with changes in antioxidant enzyme activities in a host-dependent manner under semi-solid plant growth conditions. SOD converts superoxide radicals into H_2O_2, whereas CAT and APX contribute to H_2O_2 detoxification through

complementary pathways, thereby participating in the maintenance of reactive oxygen species (ROS) homeostasis [53]. The pronounced increase in APX activity, particularly in green chiretta, suggests a differential response involving the ascorbate-dependent antioxidant pathway. However, increased antioxidant enzyme activity does not necessarily indicate the occurrence of oxidative stress or demonstrate improved ROS-scavenging capacity, as these enzymes may also be modulated as part of plant responses to beneficial microorganisms [54]. Previous studies have reported changes in SOD, CAT, and APX activities following inoculation with plant growth-promoting rhizobacteria (PGPR), supporting the involvement of antioxidant enzymes in plant responses to beneficial bacterial interactions [55]. The differences observed among green chiretta, tomato, and kale further highlight the host-dependent nature of these responses. These antioxidant enzyme results were obtained from a separate experiment using plants grown on semi-solid plant growth medium and should therefore be interpreted independently of the plant growth responses observed in the pot experiment. Furthermore, because only non-inoculated and dual-inoculated treatments were included in this assay, the observed changes cannot be attributed specifically to either bacterial strain or interpreted as evidence of synergistic effects between the two strains. Together with the transcriptional data, these findings provide complementary evidence that bacterial inoculation was associated with changes in plant defense- and stress-related responses at both the molecular and physiological levels, although the relationship between these responses and plant growth requires further investigation (Figure 9).

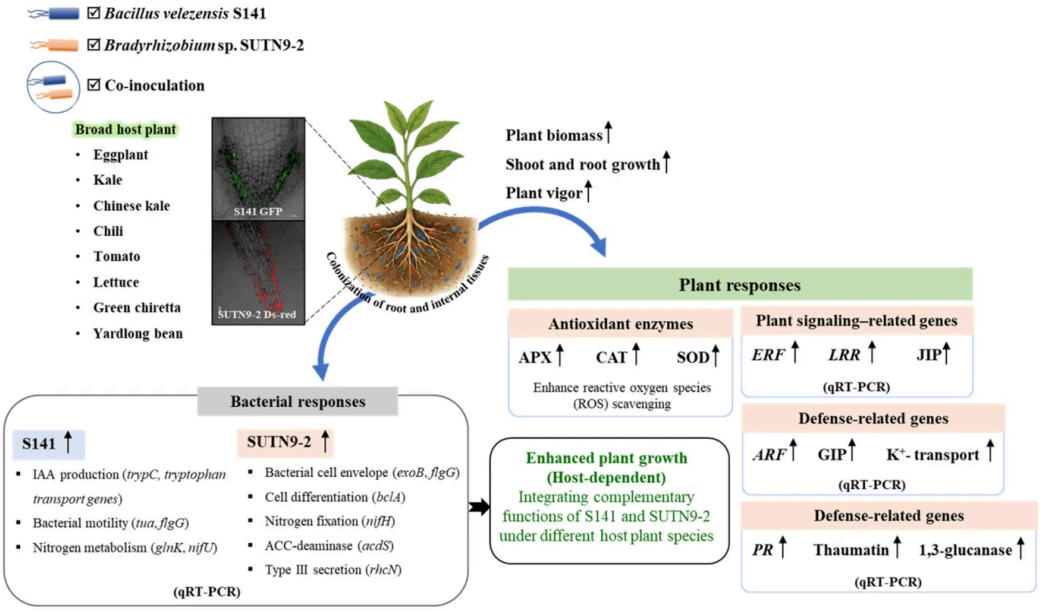


Figure 9. Proposed model of S141–SUTN9-2–plant interaction. The model integrates bacterial colonization, bacterial gene expression, plant responses (qRT-PCR), antioxidant activity, and plant growth following co-inoculation. The responses varied among host plants, highlighting the host-dependent nature of the response to S141–SUTN9-2 co-inoculation. Upward-pointing arrows indicate increases in the corresponding parameters. The illustrative element depicting a plant root system in soil with orange and blue bacteria attached to and distributed along the roots was generated using ChatGPT (GPT-4o, OpenAI) with the prompt: "Generate an illustration of a plant with its root system extending into the soil, showing orange and blue bacterial cells attached to and distributed along the roots." This AI-generated element is used for illustrative purposes only and does not represent experimental data. The conceptual model, scientific interpretation, and overall figure layout were prepared by the authors.

5. Conclusions

Taken together, the results support a host-dependent model of *Bacillus velezensis* S141–*Bradyrhizobium* sp. SUTN9-2–plant interactions, characterized by bacterial association with plant tissues and changes in bacterial gene expression, plant transcriptional responses, and plant growth. Antioxidant enzyme responses were also observed in a separate experiment under semi-solid plant growth conditions. Plant growth responses varied among the eight species and inoculation treatments. Based on dry biomass responses, dual inoculation generally showed the greatest numerical increases in six species, namely eggplant, tomato, chili, lettuce, green chiretta, and yardlong bean, whereas S141 alone produced greater numerical increases in kale and Chinese kale. These findings highlight the importance of host-dependent responses when evaluating individual and combined bacterial inoculation for potential agricultural applications.

The observed responses suggest that interactions between S141 and SUTN9-2 may involve multiple bacterial and host-associated processes. However, the underlying mechanisms remain to be established because bacterial metabolite production, plant hormone levels, direct interactions between the two bacterial strains, and nitrogen fixation activity were not directly measured. Although nodulation was observed in yardlong bean, plant nitrogen content and nitrogen transfer were not determined. Similarly, the expression of nitrogen-related genes reflects transcriptional responses rather than direct evidence of nitrogen fixation. Overall, this study provides a basis for further mechanistic investigations into how bacterial traits and host-associated factors contribute to plant responses following individual and dual inoculation, as well as for evaluating the consistency and applicability of these bacterial inoculants across diverse crops and growing conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16232415/s1. Supplementary Figure S1: . Evaluation of seed germination and batch-to-batch consistency of SUT potting soil inoculated with Bacillus velezensis S141 and Bradyrhizobium sp. SUTN9-2; Figure S2: Phenotypic characterization and nodulation efficiency of yardlong bean at 30 days after inoculation across different treatments. Figure S3: Leaf chlorophyll content (SPAD values) of eight plant species grown under pot-culture conditions at 10, 14, 20, 25, and 30 days after inoculation (DAI); Method S1. Preparation of Crude Enzyme Extracts; Method S2. Determination of Ascorbate Peroxidase (APX) Activity; Method S3. Determination of Catalase (CAT) Activity; Method S4. Determination of Superoxide Dismutase (SOD) Activity; Table S1: plant growth responses and percentage changes in dry biomass, root length, and SPAD values of eight plant species following individual and dual bacterial inoculation; Table S2: list of plant primers; Table S3: list of bacterial primers.

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