Bacillus subtilis reprograms host transcriptome and rhizosphere microbiome via systemic signaling to confer alkaline stress tolerance in garden pea

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Abstract

Soil alkalinity severely impairs legume growth. While plant-associated microbiomes can ameliorate stress, the role of *Bacillus subtilis* under alkaline stress remains unclear in garden pea. In this study, alkaline stress reduced Fe, Mn, and N content, photosystem efficiency, and biomass accumulation. Inoculation with *B. subtilis* restored micronutrient balance and improved photosynthetic parameters across genetically diverse pea cultivars. The split-root assay demonstrated that BS inoculation induces systemic signaling, which underlies alkaline tolerance in pea. Furthermore, co-culture experiments showed increased growth of both *Rhizobium leguminosarum* and *B. subtilis*, pointing to their complementary interactions that promote mutual fitness under alkaline stress. RNA□seq analysis identified 958 and 1134 differentially expressed genes upregulated and downregulated, respectively, in the roots inoculated with *B. subtilis* under alkaline conditions. The upregulated genes were mostly involved in the sugar-mediated symbiotic association (*SWEET*, *GLUT*), pH homeostasis (*cation/H+ exchanger*, *ATPase*), and nutrient assimilation (*Ammonium transporter*, *Zn/Fe permease*). Alkalinity mitigation was further assisted by increased expression of oxidoreductases, ferritin-like protein, and other redox-related genes, reflecting improved antioxidant defense and Fe storage under high pH conditions. Amplicon sequencing revealed lower richness and diversity of rhizosphere bacterial and fungal communities under alkaline stress. However, *B. subtilis* inoculation led to enrichment of beneficial taxa, such as *Pseudomonas*, *Pseudorhizobium*, *Pseudallescheria*, and *Chaetomium*, which may act as helper microbes to support nutrient cycling and enhance pea survival under alkalinity. These findings suggest that *B. subtilis* functions as a microbiome inducer, working synergistically with helper microbes to promote resilience in garden peas under alkaline stress.

Keywords: Helper microbes; nutrient assimilation; rhizosphere microbiome; soil alkalinity; sugar transport; symbiosis.

Running title: *Bacillus subtilis* promotes alkaline stress tolerance in pea plants

INTRODUCTION

Soil alkalinity is a widespread abiotic stress that severely constrains crop productivity by limiting the solubility and availability of essential nutrients, particularly Fe, Mn, and P (Wang et al., 2025; Rengasamy, 2010). In alkaline soil, high pH disrupts root membrane transport systems, impairs chlorophyll (Chl) biosynthesis, and reduces photosynthetic efficiency, ultimately resulting in stunted growth and yield reduction (Thapa et al., 2015). Legume crops are especially sensitive to alkaline stress due to their reliance on symbiotic nitrogen fixation, a process that is highly dependent on optimal nutrient uptake and balanced rhizosphere conditions (Tang et al., 2024). The increasing prevalence of alkaline soils in arid and semi-arid agricultural zones, exacerbated by irrigation with bicarbonate-rich water, underscores the urgent need for sustainable strategies to mitigate their impact (Gamalero et al., 2020).

To cope with soil alkalinity, plants employ a suite of adaptive mechanisms to maintain nutrient acquisition and cellular homeostasis. These include selective ion transport and proton pump activity to regulate the uptake of essential nutrients and cellular pH, respectively (Ku et al., 2022; Kabir et al., 2012). Another strategy involves the exudation of organic acids from roots, which acidify the rhizosphere and improve the solubility of otherwise inaccessible nutrients (Barrow & Hartemink, 2023). Morphological adaptations, such as the proliferation of deep and extensive root networks and the formation of microbial symbioses, further enhance nutrient uptake efficiency under alkaline conditions (Zhang et al., 2020; Chen et al., 2016).

Microbiome-based interventions represent a promising, environmentally sustainable strategy to strengthen plant resilience against alkalinity. Beneficial microbes influence not only local root physiology but also systemic signaling networks that coordinate stress responses at the whole-plant level (Pantigoso et al., 2022; Ortíz-Casro et al., 2009). Such signals can reprogram the root transcriptome, activate defense mechanisms, and restructure microbial communities, making plants more adaptable to stressful conditions (Jamil et al., 2022; Pantigoso et al., 2022; Ruffel et al., 2008). Among plant growth-promoting rhizobacteria (PGPR), *Bacillus subtilis* has gained significant attention for its ability to withstand environmental stresses (Sharma et al., 2023), rapid colonization of roots, and production of diverse bioactive metabolites (Mahapatra et al., 2022; Hashem et al., 2019). It facilitates the secretion of organic acids to solubilize minerals, production of siderophores to chelate Fe, synthesis of phytohormones, and modulation of antioxidant defenses (Hazarika et al., 2023; Chandwani et al., 2023). Despite these known benefits, little is understood about the roles of *B. subtills* in regulating transcriptomes and root microbiomes in the context of soil alkalinity in legumes.

The garden pea (*Pisum sativum* ssp. *hortense*), an important source of dietary micronutrients, is highly sensitive to alkaline stress (Thapa et al., 2025). Dahl et al., 2012, Elevated soil pH can severely reduce yields by disrupting photosynthetic efficiency, which manifests as leaf chlorosis and reduced plant growth (Meisrimler et al., 2016). However, the potential of *Bacillus subtilis* to alleviate alkaline stress in garden pea remains poorly understood. In this study, we investigated how *B. subtilis* inoculation modulates defense-signaling pathways, transcriptome reprogramming, and rhizosphere microbiome shifts to enhance alkaline stress tolerance in garden pea. Our findings provide novel insights into the potential of *B. subtilis*-based

bioinoculants for improving legume productivity on alkaline soils, contributing to sustainable agriculture in marginal environments.

MATERIALS AND METHODS

Plant cultivation and growth conditions

Several garden pea cultivars, such as Sugar Snap, Oregon Sugar Pod, Sugar Ann, Little Marvel, Green Arrow, and Lincoln, were used in the initial screening of genotypes. Following preliminary screening, Sugar Snap was chosen for subsequent experiments. Seeds were surface-sterilized in 2% sodium hypochlorite for 5 min, rinsed three times with sterile water, and placed in trays for germination at 25 °C for 48 h. Uniform seedlings were transplanted into pots containing 500 g of a soil mixture (field soil from a pea-growing area combined with commercial potting mix at a 1:2 ratio). The treatment groups include four conditions using lime [7.5 g $NaHCO_3$ and 4.5 g $CaCO_3$ per pot] and *B. subtilis* (1×10^{8} cfu/mL; ARS Culture Collection–USDA, NRRL B-14596): (i) control (no lime, pH ~6.5), (ii) alkaline (lime-amended soil, pH ~7.8), (iii) alkaline+BS (lime-amended soil, pH ~7.8, plus *B. subtilis* inoculum), and (iv) BS+ (no lime, pH ~6.5, plus *B. subtilis* inoculum). To maintain alkaline conditions, 15 mM $NaHCO_3$ was applied weekly throughout the 5-week growth period before data collection. For a targeted experiment, two additional treatments were included: FeEDDHA+ (lime-amended soil, pH ~7.8, supplemented with FeEDDHA at 0.5 g/500 g soil) and FeEDDHA control (non-lime soil, pH ~6.5, with FeEDDHA at 0.5 g/500 g soil). Plants were cultivated for 7 weeks prior to measurement. All experiments followed a randomized complete block design with six biological replicates per treatment, conducted in a growth chamber under a 10 h light / 14 h dark photoperiod (250 μmol m^{-2} s^{-1}) at ~25 °C. Soil physicochemical properties are provided in Supplementary Table S1.

Molecular detection of *B. subtilis* colonization in roots

DNA-based qPCR analysis was performed to study the root colonization in the roots of Sugar Snap under different growth conditions. Root samples were rinsed twice in sterile phosphate-buffered saline, briefly vortexed, and washed twice with sterile water. Genomic DNA was extracted from approximately 0.2 g of root tissue using the cetyltrimethylammonium bromide (CTAB) method (Clarke, 2009). DNA concentration and purity were determined with a NanoDrop ND-1000 spectrophotometer (Wilmington, USA), and all samples were diluted to equal concentrations prior to qPCR. Amplification was conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using *B. subtilis*-specific primers (forward: 5′-TCTGCTCGTGAACGGTGCT-3′; reverse: 5′-TTTCGCCTTATTTACTTGG-3′) (IARRPCAAS, 2013). Reactions were set up with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), with *P. sativum* GAPDH (Fw GTGGTCTCCACTGACTTTATTGGT, Rv TTCCTGCCTTGGCATCAAA) serving as the reference genes. Relative quantification of *B. subtilis* abundance was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Measurement of morphological features and chlorophyll fluorescence kinetics

Shoot height and root length were measured for each plant using a measuring tape. Shoots and roots were then harvested and oven-dried at 70 °C for 3 days to determine dry weight. For nodule assessment, roots were gently washed to remove soil debris, and nodules were carefully detached, counted under a magnifying lens, and stored at −80 °C for subsequent analyses. Chlorophyll content was determined with a handheld SPAD meter (AMTAST, USA). SPAD

readings provide a relative measure of Chl concentration in leaf tissue, reflecting photosynthetic capacity and plant health (Uddling et al., 2007). Chlorophyll fluorescence kinetics (OJIP), including the maximal photochemical efficiency of PSII (F_v/F_m) and the photosynthetic performance index (Pi_ABS), were recorded on the uppermost fully expanded leaves at three distinct points using a portable FluorPen FP 110 (Photon Systems Instruments, Czech Republic). For OJIP measurements, leaves were dark-adapted for 1 h before data acquisition.

Nutrient analysis in pea roots and leaves

Nutrient analysis was conducted on roots and young leaves. Root samples were excised, rinsed under running tap water, immersed in 0.1 mM CaSO□ for 10 min, and subsequently washed with deionized water. Young leaves were washed separately with deionized water. All samples were placed in labeled envelopes and oven-dried at 75 °C for 3 days. Elemental concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS) at the Agricultural and Environmental Services Laboratories, University of Georgia.

Determination of ammonium in roots

Ammonia concentration in root tissues was measured using Nessler's reagent spectrophotometric method (Jeong et al., 2013). Fresh root tissue (100 mg) was homogenized in 5 mL of 2% (w/v) potassium chloride, and the homogenate was centrifuged at 12,000 rpm for 10 min. One milliliter of the resulting supernatant was mixed with 1 mL of Nessler's reagent and incubated at room temperature for 15 min. Absorbance was recorded at 420 nm using a spectrophotometer. Ammonia content was calculated from a standard curve generated with known concentrations of ammonium sulfate and expressed on a fresh weight basis (μg g□¹FW).

Split-root assay

Plants were first grown for 2 weeks in sterile vermiculite prior to initiating the split-root assay in soil pots. Following transplant, split-root plants were cultivated for an additional 4 weeks before measurements were taken. The split-root setup was established using a single pot with a partitioned assembly, with modifications to the method described by Thilakarathna and Cope (2021). The taproot of each seedling was severed at the midpoint, and the lateral roots were evenly distributed between two compartments within the same pot, each filled with a 1:2 mixture of natural field soil and commercial potting mix. A solid plastic barrier was used to separate the compartments. All plants were maintained under identical growth conditions, with soil alkalinity applied according to the treatment design. In each split-root system, one compartment received *B. subtilis* inoculation, while the other remained uninoculated.

RNA-sequencing and bioinformatics analysis

RNA-seq analysis was performed on root tissues. Before RNA extraction, roots were rinsed twice with sterile water and vortexed for 10 s in sterile phosphate-buffered saline to remove surface debris. Cleaned roots were flash-frozen in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. Total RNA was extracted using the SV Total RNA Isolation System (Promega Corporation, USA). Samples with RNA integrity number (RIN) values greater than 8 were selected, and 1 μg of RNA per sample was used for library preparation. Libraries were generated with the KAPA RNA HyperPrep Kit with Poly-A Selection (Kapa Biosystems, USA) and amplified using the KAPA HiFi HotStart Library Amplification Kit (Kapa Biosystems, USA). Sequencing was conducted on an Illumina NovaSeq

6000 platform (0.2 Shared S4, 150 bp paired-end) at the RTSF Genomics Core, Michigan State University. Overall, 90.0% of reads passed quality filtering with scores ≥ Q30.

Bioinformatics processing of raw FastQ files was performed using Partek Flow genomic analysis software (Partek, St. Louis, MO, USA). Adapter sequences and low-quality reads were removed with Trimmomatic (Bolger et al., 2014) to obtain high-quality clean reads. The filtered reads were aligned to the *Pisum sativum* reference genome using HISAT2 (Kim et al., 2015), and read counts for each gene were quantified with HTSeq (Anders et al., 2015). Differentially expressed genes (DEGs) were identified using the Limma-Voom method, applying a false discovery rate (FDR) < 0.05 and an absolute log□ fold change ≥ 2 as significance thresholds. Genes with low expression (<10 normalized counts across all samples) were excluded from further analysis. Expression levels were calculated as fragments per kilobase of exon per million mapped fragments (FPKM). Venn diagrams were generated using VennDetail (Hinder et al., 2017), and heatmaps were produced with the *pheatmap* package in R (Hu et al., 2021). Functional enrichment analysis of DEGs was conducted using ShinyGO 0.76.3 (Ge et al., 2020), with gene ontology (GO) annotations obtained from EnsemblPlants (Yates et al., 2022).

Amplicon-based 16S and ITS sequencing in the rhizosphere

Rhizosphere microbial populations were characterized using Illumina amplicon sequencing, targeting the ITS region for fungi and the 16S rRNA gene for bacteria. Root samples were vortexed in sterile phosphate-buffered saline (PBS) for 10 s, rinsed twice with sterile water, and ~0.2 g of cleaned tissue was used for DNA extraction with the Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). RNase and Proteinase K treatments were included to remove RNA and protein contaminants, respectively. Amplicon libraries were generated using primer pairs 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) for the 16S rRNA gene, and ITS3 (GATGAAGAACGYAGYRAA) and ITS4 (TCCTCCGCTTATTGATATGC) for the ITS region. Sequencing was conducted on the Illumina NovaSeq 6000 platform (PE250). Raw reads were processed and trimmed with Cutadapt (Martin, 2011) and analyzed using the DADA2 pipeline (Callahan et al., 2016). Sequences identified as mitochondrial or chloroplast origin were removed. Taxonomic classification of amplicon sequence variants (ASVs) was performed using the UNITE database (Nilsson et al., 2019) for fungal ITS sequences and SILVA 138 (Quast et al., 2013) for bacterial 16S sequences. Microbial community structure was assessed by calculating Bray-Curtis distance matrices from Hellinger-transformed abundance data, followed by Principal Coordinate Analysis (PCoA) using the *phyloseq* and *vegan* packages in R. Diversity analyses included alpha and beta diversity metrics, relative abundance profiling, and non-parametric Kruskal–Wallis tests, with significance determined at $P < 0.05$ (McMurdie & Holmes, 2013).

Microbial co-culture method

Interactions between *B. subtilis* and *R. leguminosarum* were evaluated on nutrient agar under Fe-sufficient and Fe-deficient conditions, with the latter supplemented with 15 mM $NaHCO_3$ to adjust the pH to 7.8. A 5 μL inoculum of each microbial strain was cultured in three configurations: *B. subtilis* alone, *R. leguminosarum* alone, and a mixed culture of *B. subtilis* + *R. leguminosarum*. Plates were incubated for 7 days, after which colony growth was quantified using ImageJ software (National Institutes of Health, USA). The radius of each colony was

measured at three independent points, and the mean value was calculated for subsequent analysis.

Data analysis

Statistical analyses were performed using analysis of variance (ANOVA), with treatment as the independent factor. When significant differences were detected, Duncan's Multiple Range Test (DMRT) was applied for mean separation using SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). Graphs were generated using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) and the *ggplot2* package in R.

RESULTS

Screening of genotypes for tolerance to soil alkalinity with *B. subtilis*

Across genotypes, alkaline stress consistently reduced shoot height and SPAD scores compared to control plants, though the extent of recovery with *B. subtilis* varied (Table 1). In Sugar Snap and Oregon Sugar Pod, both shoot height and SPAD scores decreased significantly under alkaline stress but were restored to control levels when inoculated with *B. subtilis* with or without alkaline stress (Table 1). In Sugar Ann and Little Marvel, alkaline stress reduced both traits. Plants exposed to alkaline stress inoculated with *B. subtilis* improved shoot height, while BS+ produced the highest values in Little Marvel and Sugar Ann, exceeding the control (Table 1). In Little Marvel, SPAD scores improved under alkaline+BS and BS+ conditions, but BS+ matched control plants in Little Marvel (Table 1). For the cultivar Sugar Ann, BS+ showed higher SPAD values than the control, but the difference was not statistically significant. Green Arrow and Lincoln displayed a different pattern, with shoot height reduced under alkaline stress and remaining low under alkaline+BS conditions, but restored to control levels by *B. subtilis*. Similarly, SPAD scores declined under alkaline conditions, remained unchanged in response to *B. subtilis* under soil alkalinity, and increased significantly above control under *B. subtilis* inoculation (Table 1).

Effect of *B. subtilis* on physiological parameters and root colonization

Phenotypic observation showed that plants under alkaline had smaller shoots and roots compared to control (Fig. 1A). Plants inoculated with *B. subtilis* showed an improvement in shoot and root growth compared to alkaline stress, while plants inoculated with *B. subtilis* without stress appeared similar to control (Fig. 1A). The relative abundance of *B. subtilis* in roots was low in control and alkaline stress but significantly increased only in alkaline+BS conditions (Fig. 1B). Plant fresh weight was significantly reduced in alkaline compared to control, increased under soil alkalinity with *B. subtilis* to values comparable to control, and remained similar to the plants solely inoculated with *B. subtilis* (Fig. 1C). Further, shoot height followed the same trend (Fig. 1D). Furthermore, root length (Fig. 1E) and root FW (Fig. 1F) were significantly reduced under alkaline stress compared to control. However, both parameters were restored to control levels under alkaline+BS conditions and remained comparable to control in BS+. We also determined the root ammonium content in the roots. Root ammonium content was significantly affected by treatments. Control and BS+ plants showed the highest values, which were not significantly different from each other, while alkaline treatment had the lowest ammonium content (Fig. 1G). Plants inoculated with B. subtilis under soil alkalinity partially restored ammonium levels, resulting in significantly higher values than alkaline alone but still lower than control and BS+ conditions (Fig. 1G). We also found that F_v/F_m and PI_ABS declined significantly in alkaline

conditions compared to control, increased under alkaline stress inoculated with *B. subtilis* conditions to control levels, and remained unchanged in BS+ relative to control (Fig. 1H and 1I).

Effect of *B. subtilis* on nutrient concentrations in roots and leaves

ICP-MS analysis of nutrient contents showed a variation in their concentrations in the root and leaf tissues of garden peas grown with or without *B. subtilis* under alkaline conditions (Table 2). In alkaline-stressed plants, there was a decrease in concentrations of iron (Fe), manganese (Mn), and nitrogen (N) in both root and leaf tissues. However, the addition of *B. subtilis* to alkaline-stressed plants recovered these nutrient contents in both root and leaf tissues and was similar to control plants. Additionally, *B. subtilis* inoculated with control plants showed Fe, Mn, and N concentrations in the roots and leaves of garden peas that were similar to those in the control and alkaline + BS treatments. However, there were no significant changes in the amount of S in the root tissues under alkaline conditions compared to the control. There was a decrease in S content in leaf tissues in such conditions, and it was recovered when *B. subtilis* was added (Table 2). Similarly, there was a significant decrease in magnesium (Mg) content in roots of alkaline-stressed plants; however, there was no change in Mg content in leaf tissues under all four treatment conditions. Additionally, under alkaline conditions, calcium (Ca) concentrations in both the roots and leaves of garden peas decreased significantly, while inoculation of alkaline-stressed plants with *B. subtilis* increased Ca content in both tissues (Table 2).

Comparative performance of *B. subtilis* and inorganic Fe in alkaline soil

The effects of *B. subtilis* inoculation and inorganic Fe supplementation on pea growth under alkaline conditions were investigated and compared (Fig. 2A). The relative abundance of *B. subtilis* in roots was low in control, alkaline, alkaline+Fe, and inorganic Fe plants (Fig. 2B). In contrast, alkaline+BS conditions showed a significant increase in *B. subtilis* abundance as compared to other treatments without *B. subtilis* additions (Fig. 2B). Under alkaline stress, shoot height decreased significantly compared to the control (Fig. 2C). Inoculation with BS or supplementation with inorganic Fe under alkaline stress significantly increased shoot height relative to alkaline conditions, with values approaching those of the control. Plants inoculated with *B. subtilis* exhibited shoot height similar to controls, whereas inorganic Fe supplementation increased shoot height slightly above control values (Fig. 2C). Furthermore, SPAD scores under alkaline stress significantly reduced in relation to that in control (Fig. 2D). Both alkaline+BS and alkaline+Fe treatments restored SPAD scores to levels significantly higher than alkaline-stressed plants. Plants solely inoculated with *B. subtilis* or inorganic Fe showed similar SPAD scores to those of control plants (Fig. 2D).

Split-root assay for identifying systemic signaling

A split-root system was used to distinguish between local and systemic effects of *B. subtilis* inoculation under control and alkaline conditions (Fig. 3A–F). Root length significantly reduced in SR2 (alkaline/alkaline) compared to SR1 (control/control) conditions (Fig. 3A). Inoculation with BS in one compartment (alkaline/alkaline+BS) or both compartments (alkaline+BS/alkaline+BS) under alkaline conditions increased root length compared to SR2 (alkaline/alkaline), with no significant difference between SR3 (alkaline/alkaline+BS) and SR4 (alkaline+BS/alkaline+BS). In control conditions, BS inoculation in one (control+BS/control+BS) or both compartments (control/control+BS) did not significantly change root length relative to SR1 (Fig. 3A). Root FW, shoot height, shoot FW, and F_v/F_m all

showed a similar trend: values were significantly reduced in SR2 (alkaline/alkaline) compared to SR1 (control/control), while BS inoculation in one (SR3) or both (SR4) compartments under alkaline stress significantly improved these traits relative to SR2, with no differences between SR3 and SR4 (Fig. 3B–D, F). In control conditions (SR1, SR5, and SR6), these parameters remained unchanged. SPAD score followed a related pattern, with significant reduction in SR2 (alkaline/alkaline) compared to SR1 (control/control), partial recovery in SR3 (alkaline/alkaline+BS), and the highest values in SR4 (alkaline+BS/alkaline+BS) under alkaline stress, while control treatments showed no differences (Fig. 3E). No significant differences were observed between compartments within the same split-root treatment (Fig. 3E).

Interaction between *B. subtilis* and *R. leguminosarum* in co-culture

The effect of co-culturing *B. subtilis* with *R. leguminosarum* was assessed under both control and alkaline conditions over three, five, and seven days (Fig. 4A-4B). Under non-alkaline conditions, colony sizes of *R. leguminosarum* and *B. subtilis* increased progressively over time in both single and co-culture treatments. On day three, no significant differences in colony size were observed between single and co-culture for either species. By day five and day seven, both species exhibited larger colony diameters compared to day three, but differences between single and co-culture remained statistically non-significant (Fig. 4A). Under alkaline conditions, colony sizes of both *R. leguminosarum* and *B. subtilis* were consistently smaller compared to control pH across all time points. On day three, growth reduction was evident in both species relative to their respective control pH counterparts, and coculture significantly altered the colony size of *R. leguminosarum* with an increase in colony area (Fig. 4B). Similar patterns were observed on days five and seven, with gradual increases in the colony diameter of *R. leguminosarum* over time, and significant differences between the single- and co-culture treatments under alkaline stress. However, there were no changes in colony growth of *B. subtilis* over three, five, and seven growing days between single and co-culture conditions (Fig. 4B). Overall, colony expansion for both species was slower in alkaline conditions than in control pH, and co-culturing confers a measurable growth advantage for only *R. leguminosarum* under alkaline conditions (Fig. 4B).

Transcriptomic changes in roots

Principal coordinate analysis (PCoA) based on transcriptome profiles showed clear separation among the treatments (Fig. 5A). Particularly, control and BS+ clustered closely together, while alkaline and alkaline+BS conditions formed distinct clusters, indicating strong transcriptional shifts due to alkaline stress and *B. subtilis* inoculation (Fig. 5A). In Venn diagram analysis, alkaline+BS treatment had a unique set of upregulated (958) and downregulated (1134) genes in the roots compared to alkaline stress (Fig. 5B-5C). Specifically, alkaline+BS exhibited a large number of uniquely upregulated genes not shared with alkaline or BS+, along with a smaller subset of genes overlapping with control-related profiles (Fig. 5A). In contrast, BS+ showed fewer DEGs compared to control, suggesting minimal transcriptomic alteration in non-stress conditions (Fig. 5A). Heatmap visualization (Fig. 5D–G) highlighted key functional categories of differentially expressed genes (DEGs). In the alkaline+BS treatment, numerous genes related to symbiotic association (e.g., sugar phosphate transporter, glucose transporter GLUT, Sugar transporter SWEET, MF sugar transporter–like, Sugar transport protein STP, UDP–sugar pyrophosphorylase) were significantly upregulated compared to alkaline stress (Fig. 5D; Supplementary Table S2). Genes associated with nutrient transport and assimilation (e.g.,

ammonium transporter, nitrite and sulphite reductase, inorganic pyrophosphatase, amino acid transporter, Cys/Met metabolism, Iron/zinc purple acid phosphatase, malic oxidoreductase, SulP transporter, Zinc/iron permease, MFS transporter) were also more highly expressed in alkaline+BS compared to alkaline stress (Fig. 5E). Under alkaline conditions without *B. subtilis*, these genes generally exhibited reduced expression compared to control plants (Fig. 5E). Furthermore, redox and osmotic adjustment genes (e.g., glutaredoxin, oxidative stress regulator, glutathione synthase, thioredoxin, glutathione *S*-transferase, ferritin-like superfamily, dehydrin, aquaporin transporter) were induced in alkaline+BS relative to the plant solely stressed with alkaline (Fig. 5F). Lastly, several genes (*isoprenoid synthase*, *ethylene-responsive TF*, *cation/H+ exchanger*, *ABC-2 type transporter*, *ABC transporter type 1*, *root meristem growth factor 3*, *AP2/ERF ethylene responsive factor*, *oxoglutarate*, *small auxin-up RNA*, *ATPase*) related to growth and stress response showed significant upregulation due to B. subtilis under soil alkalinity related to alkaline soil (Fig. 5G; Supplementary Table S2).

Gene ontology enrichment analysis

Gene ontology (GO) enrichment analysis was conducted to compare the transcriptome profiles in the roots of pea plants cultivated under alkaline stress with or without *B. subtilis* (Fig. 6A-B). In the upregulated gene set, soil alkalinity inoculated with *B. subtilis* showed significant enrichment in biological process (BP) categories, including response to water, response to acid chemical, response to inorganic substances, and organic acid catabolic process. Molecular function (MF) terms enriched in this group included oxidoreductase activity, transmembrane transporter activity, heme binding, and ammonia-lyase activity. Cellular component (CC) terms were enriched for intracellular organelle and membrane-bound organelle, indicating broad transcriptional activation of stress-response and transport-related pathways in the presence of BS under alkaline stress (Fig. 6A). In contrast, the downregulated gene set in alkaline+BS conditions relative to alkaline was enriched for BP categories such as defense response, regulation of metabolic process, and regulation of biosynthetic process. MF terms were dominated by oxygen binding, iron binding, and catalytic activity associated with primary metabolism, while CC terms showed enrichment for the extracellular region and cell wall (Fig. 6B).

Rhizosphere microbial community structure

Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity showed plants cultivated in non-stressed conditions with or without *B. subtilis* clustered together (Fig. 7A). Also, alkaline and alkaline+BS formed distinct clusters, reflecting the effect of alkaline stress and BS inoculation on bacterial assemblages (Fig. 7A). Alpha diversity analysis revealed significant differences in observed richness and Simpson diversity index among treatments (Fig. 7B). Observed richness showed significant variations, while Simpson diversity index showed no changes among the treatment groups (Fig. 7B). At the family level, *Pseudomonadaceae*, *Gemmatiomonadaceae*, and *Flavobacteriaceae* showed statistically significant differences in relative abundance among the treatments (Fig. 7C). *Pseudomonadaceae* and *Flavobacteriaceae* were markedly enriched in the rhizosphere of plants inoculated with *B. subtilis* under soil alkalinity compared to alkaline stress only. Other dominant families such as *Gemmatiomonadaceae*, *Rhizobiaceae*, and *Sphingomonadaceae* exhibited treatment-dependent shifts but without statistical significance (Fig. 7C). At the genus level, *Variovorax*, *Shinella*, *Pseudorhizobium*, *Pseudomonas*, *Novosphingobium*, *Flavobacterium*, *Brevundimonas*, and *Bosea* displayed statistically significant variation across treatments (Fig. 7D). Particularly,

Pseudomonas and *Pseudorhizobium* showed a significant enrichment in alkaline-exposed conditions inoculated with *B. subtilis* relative to the conditions solely exposed to soil alkalinity. In contrast, *Shinella* was enriched under alkaline stress, while *Bosea* and *Variovorax* were predominant in control plants (Fig. 7D).

In this study, PCoA of ITS showed distinct clustering of rhizosphere fungal communities among the treatment groups (Fig. 8A). Particularly, control and BS+ grouped closely, indicating minimal differences in fungal composition under non-stress conditions. In contrast, alkaline and alkaline+BS occupied separate positions from control, with alkaline+BS partially shifting toward the control/BS+ cluster, indicating that *B. subtilis* inoculation under alkaline stress altered fungal community structure (Fig. 8A). In our analysis, observed richness and Simpson diversity index showed no significant changes due to B. subtilis in the presence or absence of soil alkalinity (Fig. 8B). Among the families, *Halosphaeriaceae* and *Cladosporiaceae* exhibited lower relative abundance, whereas *Chaetomiaceae* showed higher abundance under soil alkalinity inoculated with *B. subtilis* compared to alkaline stress (Fig. 8C). In contrast, the dominant family *Aspergillaceae* displayed broadly similar abundance between the two treatments. Among significant genera, *Pseudallescheria* and *Chaetomium* showed significant enrichment in the rhizosphere of plants inoculated with *B. subtilis* under soil alkalinity compared to alkaline stress (Fig. 8D).

DISCUSSION

Soil alkalinity is a major constraint to legume productivity, primarily due to reduced availability of essential micronutrients (Gong et al., 2020). In this study, alkaline stress significantly disrupted pea performance across multiple physiological scales from nutrient acquisition to canopy chlorophyll status and photosystem function, leading to reduced biomass accumulation. Inoculation with *B. subtilis* effectively alleviated these negative impacts, restoring nutrient uptake, improving photosynthetic efficiency, and enhancing biomass accumulation. The underlying resilience mechanism appears to be multifactorial, encompassing direct nutrient mobilization, systemic signaling, and rhizosphere microbiome restructuring, suggesting *B. subtilis* as a holistic modulator of pea performance under high pH conditions.

B. subtilis rebalances ion homeostasis and photosynthetic efficiency under alkaline stress

Alkaline stress reduced Fe and Mn concentrations in both roots and leaves, aligning with the reduced solubility and impaired Strategy I Fe acquisition in high-pH soils (Kim and Guerinot, 2007). Inoculation with *B. subtilis* reversed these deficits, bringing Fe and Mn back to control levels in both organs, alongside improvement in Ca and total N contents. These ionomic recoveries paralleled gains in plant biomass and photosynthetic parameters, pointing to the coordinated restoration of resource supply and photochemical capacity. Such recovery aligns with established traits of *B. subtilis* relevant to alkaline soils, including siderophore and organic acid production, and antioxidant pathway activation (Kloepper et al., 2004; Egamberdieva et al., 2017). The fact that similar benefits occurred across genetically diverse pea cultivars, albeit with variable magnitudes, reflects the common but genotype-dependent nature of PGPR–legume interactions (Hasan et al., 2025; Vejan et al., 2016). Overall, these findings demonstrate that *B. subtilis* is highly effective in restoring micronutrient balance and photosynthetic performance in legumes under alkaline stress.

Inorganic Fe supplementation negates the physiological need for *Bacillus*-driven rescue

The FeEDDHA supplementation study offered mechanistic control by directly supplying a highly stable Fe-chelate capable of maintaining Fe solubility at high pH (Rojas et al., 2008). When FeEDDHA was applied to alkaline soil, plants achieved control-like growth and Chl levels without *B. subtilis* inoculation. This absence of additional benefit from *B. subtilis* under alkaline conditions demonstrates that the bacterium's primary contribution to alkaline soil lies in alleviating Fe deficiency. Once Fe limitation was chemically bypassed, the physiological necessity for *B. subtilis*-driven nutrient mobilization was removed. Alkaline soils can impair root growth, thereby reducing the efficient absorption of essential minerals by plants (Saleem et al., 2023). While FeEDDHA remains stable under alkaline conditions, a portion of the Fe can still bind to soil particles, decreasing its bioavailability and overall effectiveness (Zuluaga et al., 2023). These findings suggest a complementary role for microbial inoculants and inorganic Fe supplementation. FeEDDHA offers rapid correction of acute Fe deficiency, while *B. subtilis* provides a sustainable, broader-spectrum improvement that also benefits Mn and Ca uptake, photosynthetic resilience, and microbiome health. In low-input or organic systems, *B. subtilis* may thus serve as a viable alternative to synthetic chelates, avoiding their higher costs and potential environmental persistence.

Systemic signaling reveals a whole-plant response

The split-root assay demonstrated that *B. subtilis* inoculation in only half of the root system conferred comparable benefits to uninoculated compartments, indicating that the effects extended beyond the local rhizosphere and involved systemic signaling. These responses point toward the generation of mobile molecular signals, potentially including phytohormones and secondary metabolites that travel through the vascular system to modulate physiology in distant root zones and shoots (Martínez-Medina et al., 2013). The observed improvements in plant biomass and photosynthetic efficiency in both inoculated and distal root zones suggest that *B. subtilis* triggers a whole-plant acclimation program. This likely involves the integration of root-derived signals into shoot-level metabolic adjustments and photosynthetic optimization, ensuring coordinated nutrient uptake and energy production. Such long-distance communication resembles induced systemic tolerance reported for other plant PGPR, where signal perception in one root region primes the rest of the plant for improved stress resilience (Conrath et al., 2006; Hartmann et al., 2021).

To further assess whether *B. subtilis* enhances N_2 fixation via direct compatibility with *R. leguminosarum*, we performed co-culture assays under Fe-sufficient and Fe-deficient conditions at pH 7.8. The co-culture experiments in Fe-sufficient plates revealed no significant differences in colony expansion between *B. subtilis*, *R. leguminosarum*. However, their mixed cultures at high pH showed growth enhancement, indicating that the synergistic effects observed in planta are likely to arise from complementary interactions in alkaline media. Consistent with this, improved Fe and N status supported by increased ammonium transport in *B. subtilis*-treated roots under alkaline stress suggests that these benefits are alkaline-dependent. This plant-mediated facilitation aligns with previous reports in PGPR–rhizobia systems, where enhanced symbiotic performance under stress relies more on the host's metabolic and signaling reprogramming rather than on direct microbe–microbe interactions (Frey-Klett et al., 2011; Vishwakarma et al., 2020).

This whole-plant response is consistent with transcriptomic enrichment in transporter and signaling genes that likely mediate these distal effects. Moreover, the complementarity and mutualistic reinforcement between *B. subtilis* and *R. leguminosarum* may facilitate higher rhizobial abundance and a more stable microbiome assembly, contributing to sustained pea performance under alkaline conditions.

Transcriptomic reprogramming improves nutrient acquisition and redox homeostasis

RNA-seq analysis revealed that *B. subtilis* inoculation under alkaline stress triggered a broad transcriptional reprogramming in pea roots, with pronounced upregulation of genes associated with symbiotic association, mineral acquisition, phytohormone signaling, and redox regulation. The upregulation of multiple sugar transporter genes under alkaline stress suggests an active role of carbohydrate allocation and exchange due to symbiotic association with *B. subtilis*. These transporters likely facilitate bidirectional sugar movement between the host and symbiotic partners mediated by *B. subtilis*, ensuring an adequate supply of carbon for microbial metabolism while maintaining host energy balance. Particularly, SWEET sugar transporters not only facilitate carbon allocation but also modulate nodule functioning and microbial colonization (Chen et al., 2023; Breia et al., 2021). Furthermore, enrichment of transporter-related transcripts, including those for Fe, Mn, nitrate, and phosphate uptake, suggests an enhanced capacity for nutrient mobilization and assimilation under conditions where availability is otherwise restricted by high pH (Lucena, 2006; Kobayashi & Nishizawa, 2012). Ion transport–related genes, including cation/H□ exchanger (*PSAT3G088320*) and ATPase (*PSAT7G218840*), were also elevated, likely contributing to pH homeostasis and nutrient uptake under alkaline stress (Wang et al., 2014).

Alkali-exposed plants inoculated with *B. subtilis* showed the upregulation of several sugar transport–related genes (*SWEET*, *GLUT)*. It indicates that *B. subtilis* enhances carbon allocation to support symbiotic functions under alkaline stress. Efficient sugar trafficking to nodules and rhizosphere microbes (Lei et al., 2025) and symbiotic associations (Pieterse et al., 2014; Zamioudis & Pieterse, 2012). Such reprogramming of carbohydrate transport pathways aligns with reports that PGPR like *B. subtilis* stimulate sugar-mediated nutrient exchange to sustain nitrogen fixation and plant resilience under high pH conditions (Berendsen et al., 2018; Kryvoruchko et al., 2018). This may be linked to the upregulated genes for ammonium transport (*PSAT0S1495G0040*), amino acid transport (*PSAT7G178640*), and Cys/Met metabolism (*PSAT6G052400*) that are associated with nitrogen and sulfur assimilation, critical for stress adaptation (Hasan et al., 2025). ABC transporters (*PSAT2G058680*, *PSAT3G053400*) and oxoglutarate-related metabolism (*PSAT6G045440*) may further support metabolite exchange and carbon–nitrogen balance, as previously reported for legumes under symbiotic and abiotic stress conditions (Khan et al., 2020).

Furthermore, there was a strong induction of genes involved in redox homeostasis and osmotic adjustment, suggesting that *B. subtilis* enhances antioxidant defense and water balance under alkaline stress. Similarly, the oxidative stress regulator suggests coordinated activation of stress-responsive transcriptional programs, in agreement with other findings that PGPR treatments can activate redox signaling networks in legumes (Al-Turki et al., 2023; Ahmad et al., 2022). Upregulation of oxidoreductase genes likely bolsters the plant's oxidative defense, enabling protection of cellular structures and photosynthetic machinery from oxidative damage

(Choudhury et al., 2017). Elevated ferritin-like protein transcripts (*PSAT6G040040*) indicate improved Fe storage and mitigation of Fe-induced oxidative bursts (Hasan et al., 2025). In parallel, there was a suppression of defense-associated transcripts, indicating a strategic reallocation of resources away from constitutive defense toward growth and nutrient acquisition, a hallmark of induced systemic tolerance (Conrath et al., 2006). This transcriptional profile aligns with the systemic effects observed in the split-root assay, reinforcing the idea that *B. subtilis* elicits long-distance signaling that primes the plant for improved nutrient uptake and growth under alkaline stress, while minimizing the metabolic cost of constant defense activation. Such a coordinated molecular shift is consistent with the multifaceted benefits of *B. subtilis* inoculation in garden pea, where stress tolerance and symbiotic compatibility converge to enhance overall plant performance under alkaline stress conditions.

Microbiome restructuring as an ecological layer of stress resilience

The PCoA analysis of bacterial communities demonstrated that *B. subtilis* inoculation modulated rhizosphere assemblages under alkaline stress, leading to distinct clustering patterns across treatments. Soil alkalinity reduced bacterial richness, which was partially restored by *B. subtilis* inoculation. It suggests that *B. subtilis* mitigates this niche contraction, possibly by creating microenvironments conducive to diverse microbial colonization (Egamberdieva et al., 2017). The similarity of bacterial community structure in non-stress conditions with or without *B. subtilis* suggests that *B. subtilis* does not disrupt native bacterial networks in healthy soils but rather coexists within the established community (Hartman & Tringe, 2019). In contrast, alkaline stress induced a substantial shift in community composition, likely driven by high pH-mediated alterations in nutrient availability for stress-tolerant taxa (Rousk et al., 2010). Importantly, the partial restoration of the microbial profiles under soil alkalinity toward the control cluster implies that *B. subtilis* inoculation rebalanced the microbial network under stress, potentially via root exudate modulation and production of metabolites that favor beneficial taxa (Gong et al., 2023). At the genus level, the enrichment of *Pseudomonas* and *Pseudorhizobium* influenced by *B. subtilis* under soil alkalinity aligns with their documented plant growth–promoting traits, including production of phytohormones, phosphatases, and siderophores (Compant et al., 2019; Glick, 2014). *Pseudomonas* spp. is well-known for producing siderophores and phytohormones under alkaline conditions (Lemare et al., 2022; Sainz-Mejías et al., 2020). Furthermore, studies demonstrate that fluorescent pseudomonads secrete high-affinity siderophores (pyoverdines) that mobilize Fe and can be especially beneficial under alkaline or Fe-limiting soils (Cornelis and Matthijs, 2007; Kümmerli, 2022). Many strains solubilize inorganic phosphate via the PQQ-dependent gluconic-acid pathway and phosphatases, improving P uptake and root growth (Lugtenberg & Kamilova, 2009). *Pseudorhizobium* is a genus separated from Rhizobium by phylogenomics, with isolates from seawater, rocks, and polluted soils, adapted to harsh environments (Lassalle et al., 2021). Although its effects on crops are less documented, *Pseudorhizobium's* contribution to plant performance likely stems from metal/metalloid transformation and stress-tolerant physiology (Lassalle et al., 2021; Andres et al., 2013). The enrichment of *Klebsiella*, and *Brevundimonas* due to *B. subtilis* under non-stress conditions points toward enhanced functional redundancy in the rhizosphere, which could buffer against future environmental perturbations. Collectively, these results suggest that *B. subtilis* does not simply act as a single inoculant but rather as a microbiome engineer, reshaping other beneficial bacterial networks to support nutrient acquisition, stress resilience, and host–microbe symbiotic stability under soil alkalinity.

Similarly, ITS-based fungal community analysis revealed a parallel restorative effect of *B. subtilis* on fungal assemblages. Alkaline stress reduced fungal richness and diversity, consistent with the suppression of pH-sensitive saprophytic and mutualistic fungi (Lauber et al., 2009). Although richness and diversity did not differ significantly, fungal community composition shifted toward that of control treatments with BS inoculation. *Pseudallescheria* (teleomorph of Scedosporium) is best supported as a saprotroph that degrades lignin and aromatics, processes that can indirectly enhance nutrient turnover in soils (Poirier et al., 2023). Also, *Pseudallescheria* detected in plant tissues may reflect transient environmental contamination rather than stable, beneficial endophyte status (Zhang et al., 2023). However, *Chaetomium* can act as a beneficial endophyte/biocontrol (Feng et al., 2023). It can also trigger induced systemic resistance (ISR) since transcriptomic work in tomato shows JA/ET-linked immune priming after *C. globosum* treatment (Singh et al., 2021). Beyond defense, *C. globosum* mobilizes phosphorus via secreted phosphatases/phytases, increasing crop P uptake and yield in wheat and pearl millet (Tarafdar and Gharu, 2006). Hence, the enrichment of *Pseudallescheria* and *Chaetomium* by B. subtilis under alkaline soil may provide indirect benefits to pea plants by accelerating decomposition and recycling of nutrients.

CONCLUSIONS

Our findings demonstrate that *B. subtilis* inoculation effectively mitigates the adverse effects of alkaline stress in garden pea by restoring Fe, Mn, and N levels, improving photosystem efficiency, and increasing biomass across diverse cultivars (Fig. 9). Split-root assays indicated the involvement of systemic mobile signaling, while co-culture experiments revealed complementary interactions between *B. subtilis* and *Rhizobium*. Transcriptome profiling further showed strong induction of genes associated with sugar transport, pH homeostasis, and nutrient assimilation, together with elevated expression of oxidoreductases and ferritin-like protein, suggesting improved antioxidant defense and Fe storage. In parallel, *B. subtilis* enriched beneficial taxa, such as *Pseudomonas*, *Pseudorhizobium*, and *Chaetomium*, which may function as helper microbes to reinforce stress tolerance under alkaline conditions. Collectively, these findings highlight how *B. subtilis* orchestrates molecular, physiological, and microbiome-level reprogramming, establishing its potential as a targeted bioinoculant for sustaining legume productivity in alkaline soils.

Data availability

Illumina sequencing data were submitted to NCBI under the following accession numbers: PRJNA1313627 (RNA-seq), PRJNA1313634 (16S) and PRJNA1313644 (ITS).

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Author contributions

AHK conceived the study, performed the molecular experiments, analyzed the data, performed bioinformatics analysis and drafted the manuscript. AT cultivated the plants and performed split-root assay. MRH performed microbial co-culture experiments and extracted the DNA. MGM interpreted the results and critically revised the manuscript.

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Table 1. Genotypic screening of garden pea capable of utilizing *B. subtilis* (BS) to cope with soil alkalinity. Data represent means ± SDs of three independent biological samples. Statistically significant differences in the ANOVA test among different treatments are indicated by different alphabetical letters at the $P < 0.05$ level.

Genotypes	Parameters	Control	Alkaline+	Alkaline + BS	BS+
Sugar Snap	Shoot height (cm)	82±2.5a	56±3.2b	78±1.5a	83±4.1b
	SPAD score	33±0.76a	23±2.17b	32±2.47a	34±1.86a
Oregon Sugar Pod	Shoot height (cm)	24±1.7a	14±3.3b	22±1.8a	25±0.57a
	SPAD score	33±1.4a	23±1.9b	34±2.3a	34±2.2a
Sugar Ann	Shoot height (cm)	26±2.0a	21±0.57b	24±0.57a	32±3.6ac
	SPAD score	39±1.9a	33±2.7b	36±1.7b	36±2.4b
Little Marvel	Shoot height (cm)	15±0.90a	7±2.4b	12±1.3a	42±5.8c
	SPAD score	47±2.0a	31±1.8b	36±2.2c	44±2.5a
Green Arrow	Shoot height (cm)	24±1.1a	19±1.9b	20±1.7b	28±3.0a
	SPAD score	47±1.7a	39±1.9b	42±1.5b	48±1.3a
Lincoln	Shoot height (cm)	19±1.0a	15±0.57b	14±2.0b	19±1.5a
	SPAD score	34±0.36a	30±1.5b	31±1.5b	37±3.8a

Table 2. Changes in nutrient concentrations in roots and leaves of pea plants inoculated with or without *B. subtilis* (BS) under control and alkaline soil conditions. Data represent means ± SDs of three independent biological samples. Statistically significant differences in the ANOVA test among different treatments are indicated by different alphabetical letters at the $P < 0.05$ level.

Elements	Roots				Leaves			
	Control	Alkaline	Alkaline+BS	BS+	Control	Alkaline	Alkaline+BS	BS+
Fe (ppm)	1011±72.8a	660±96.8b	997±130.4a	999±42.1a	202±33.1a	137±22.0b	205±17.6a	196±23.2a
Mn (ppm)	70±5.5a	39±11.6b	100±25.0a	114±27.3a	49±7.1a	31±3.6b	37±5.8a	46±10.6a
S (%)	0.10±0.01a	0.09±0.01a	0.10±0.005a	0.09±0.01a	0.22±0.3a	0.13±0.01b	0.23±0.02a	0.24±0.01a
Mg (%)	0.29±0.05a	0.16±0.01b	0.36±0.07a	0.28±0.05a	0.15±0.2a	0.09±0.01a	0.14±0.02a	0.16±0.03a
Ca (%)	0.76±0.08a	0.51±0.04b	1.01±0.03a	0.99±0.09a	0.28±0.03a	0.18±0.02b	0.49±0.06c	0.53±0.11c
N (%)	3.2±0.26a	2.4±0.18b	3.2±0.22a	3.4±0.57a	7.0±1.19a	5.5±1.33b	7.1±0.68a	7.6±1.04a

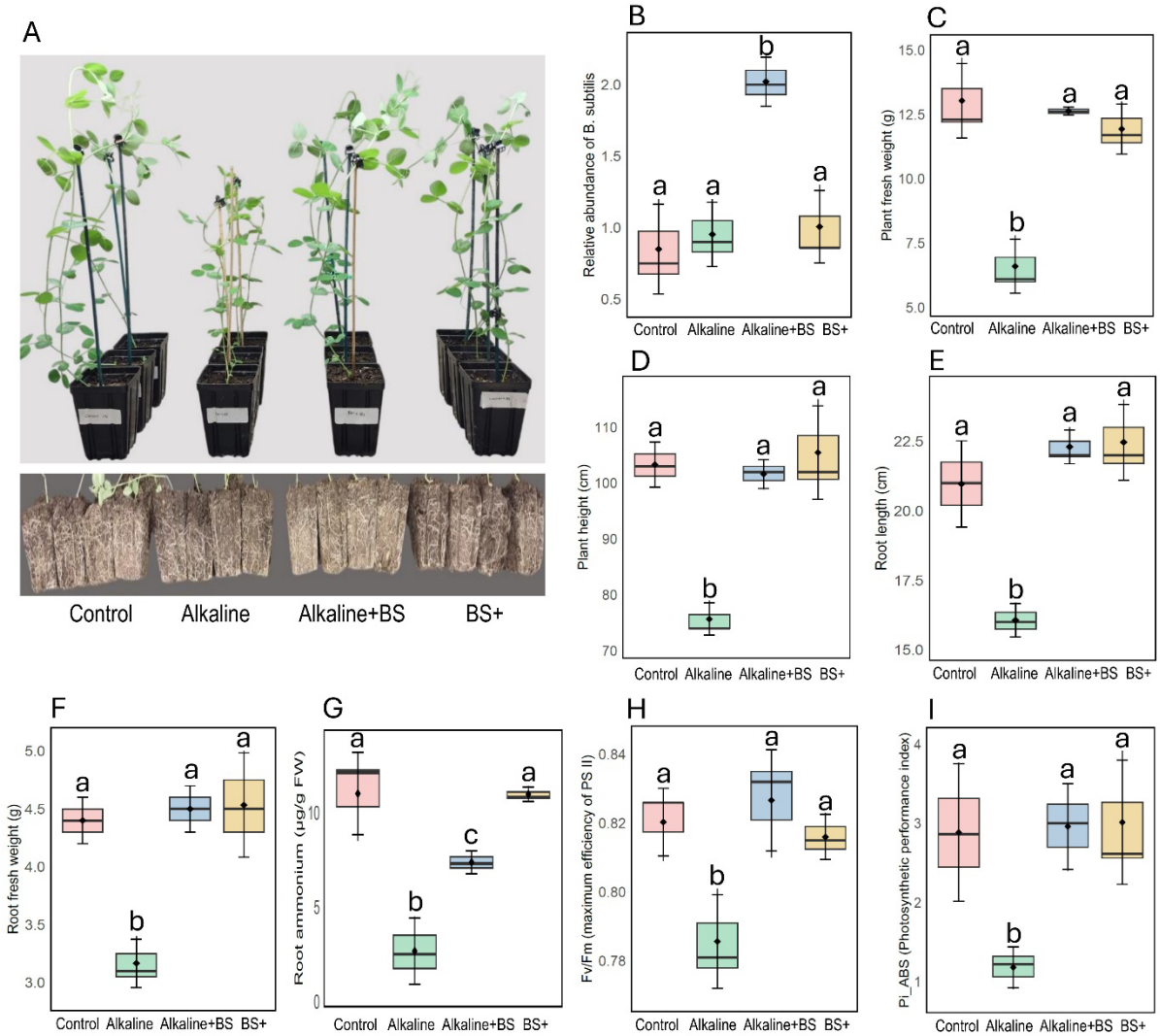

Fig. 1. Effect of *B. subtilis* (BS) on plant phenotypes (A), relative abundance of *B. subtilis* (BS) in the roots (B), plant fresh weight (C), plant height (D), root length (E), root fresh weight (F), root ammonium content (G), F_v/F_m: maximum efficiency of PSII (H), and PI_ABS: photosynthetic performance index (I) in pea plants grown under four treatments: control, alkaline, alkaline+BS, and BS+. Data represent means ± SDs of three biological replicates (n = 3). Different letters above the box plots indicate statistically significant differences among treatments ($p < 0.05$, one-way ANOVA followed by post hoc test).

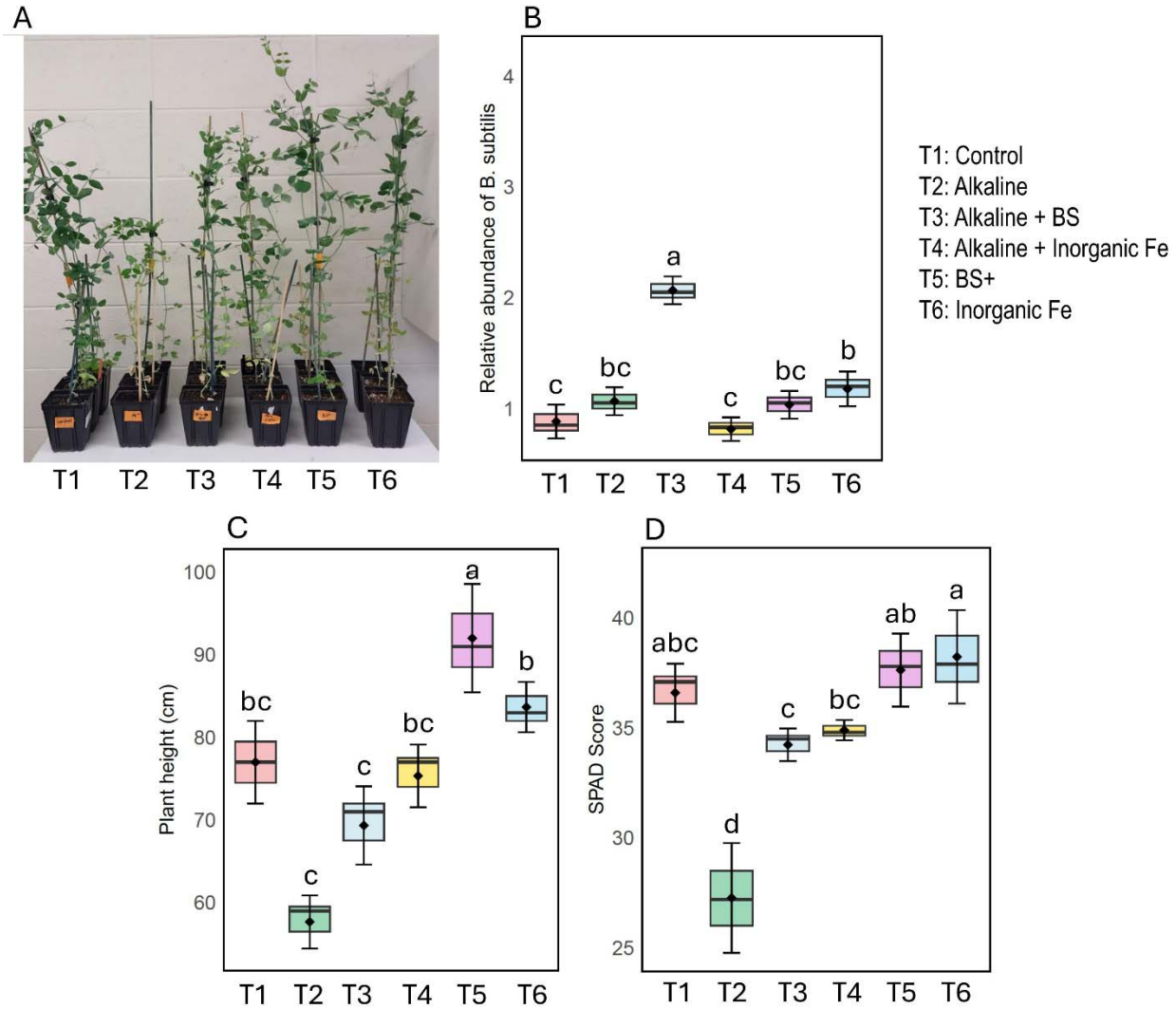

Fig. 2. Growth performance and physiological responses of pea plants under different treatments (T1: control, T2: alkaline, T3: alkaline + *B. subtilis* BS, T4: alkaline + inorganic Fe, T5: BS+, and T6: inorganic Fe) on (A) phenotype of plants from each treatment, (B) relative abundance of *B. subtilis* in the roots, (C) plant height, and (D) leaf SPAD score. Data represent means ± SDs of three biological replicates (n = 3). Different letters above the box plots indicate statistically significant differences among treatments ($p < 0.05$, one-way ANOVA followed by post hoc test).

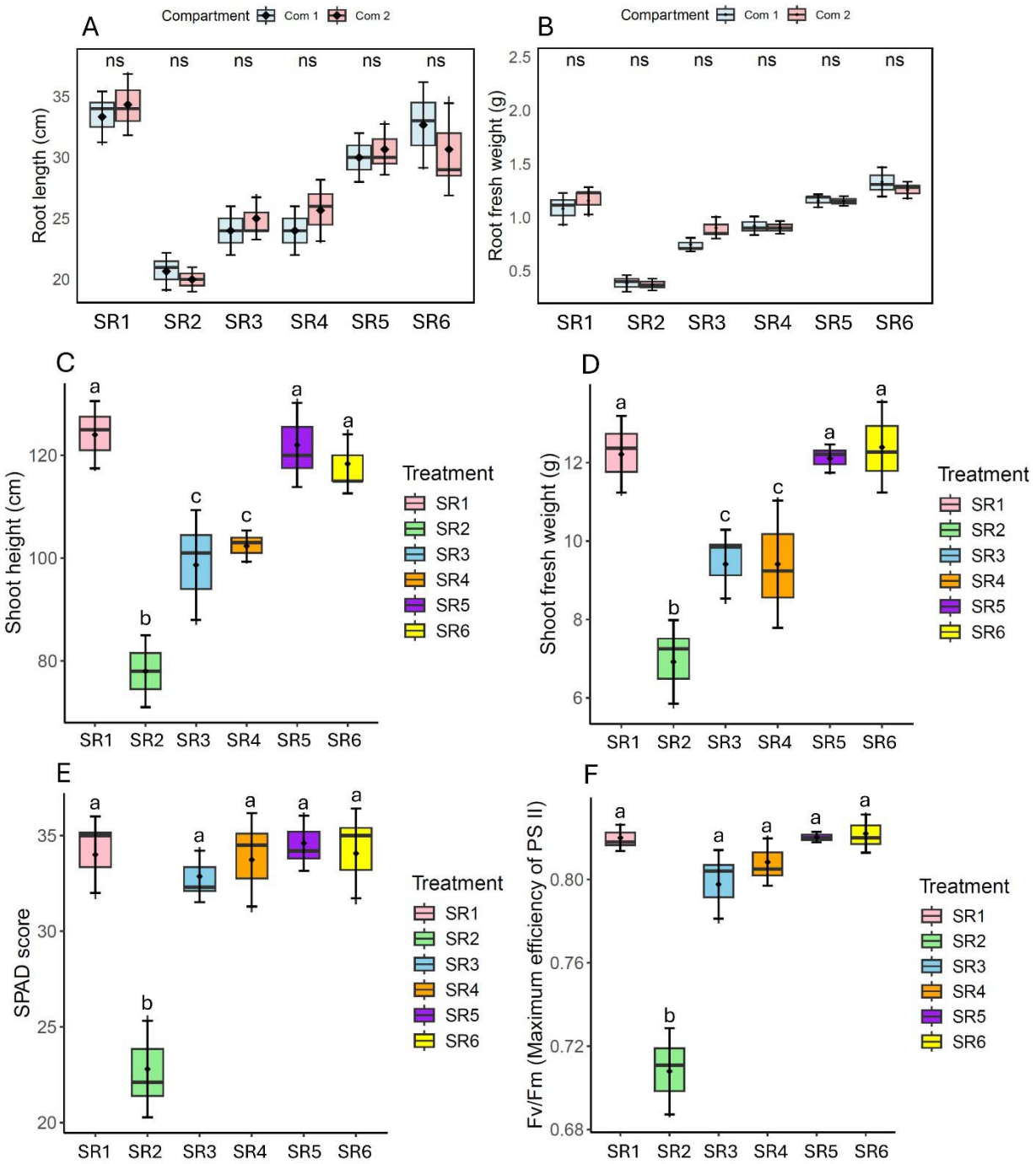

Fig. 3. Effects of different split-root treatments (SR1: control/control, SR2: alkaline/alkaline, SR3: alkaline/alkaline+BS, SR4: alkaline+BS/alkaline+BS, SR5: control/control+BS, SR6: control+BS/control+BS) on root length (A), root fresh weight (B), shoot height (C), shoot fresh weight (D), SPAD score (E), and Fv/Fm: maximum efficiency of PSII (F) of pea plants cultivated in two compartments (com 1 and com 2). Data are means ± SDs from three biological replicates (n = 3), with letters indicating statistical differences based on one-way ANOVA followed by post hoc tests ($p < 0.05$).

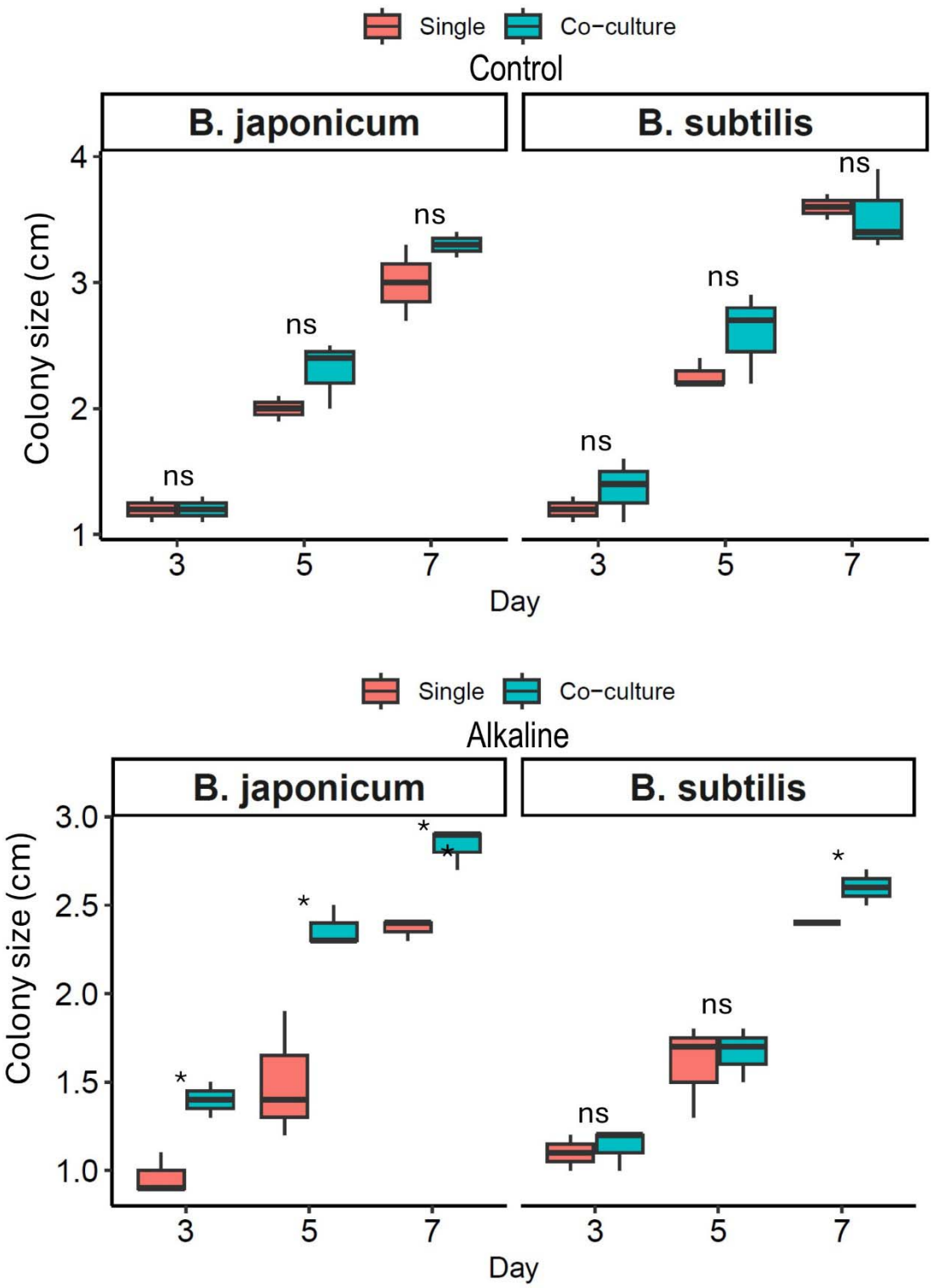

Fig. 4. Effect of single and co-culture growth on colony size of *R. leguminosarum* and *B. subtilis* under control (A) and alkaline conditions (B) over 3, 5, and 7 days. Data are means ± SDs from three biological replicates (n = 3), with significance (* = $P < 0.05$, ns = not significant) determined by t-test comparison between single and co-culture within each time point.

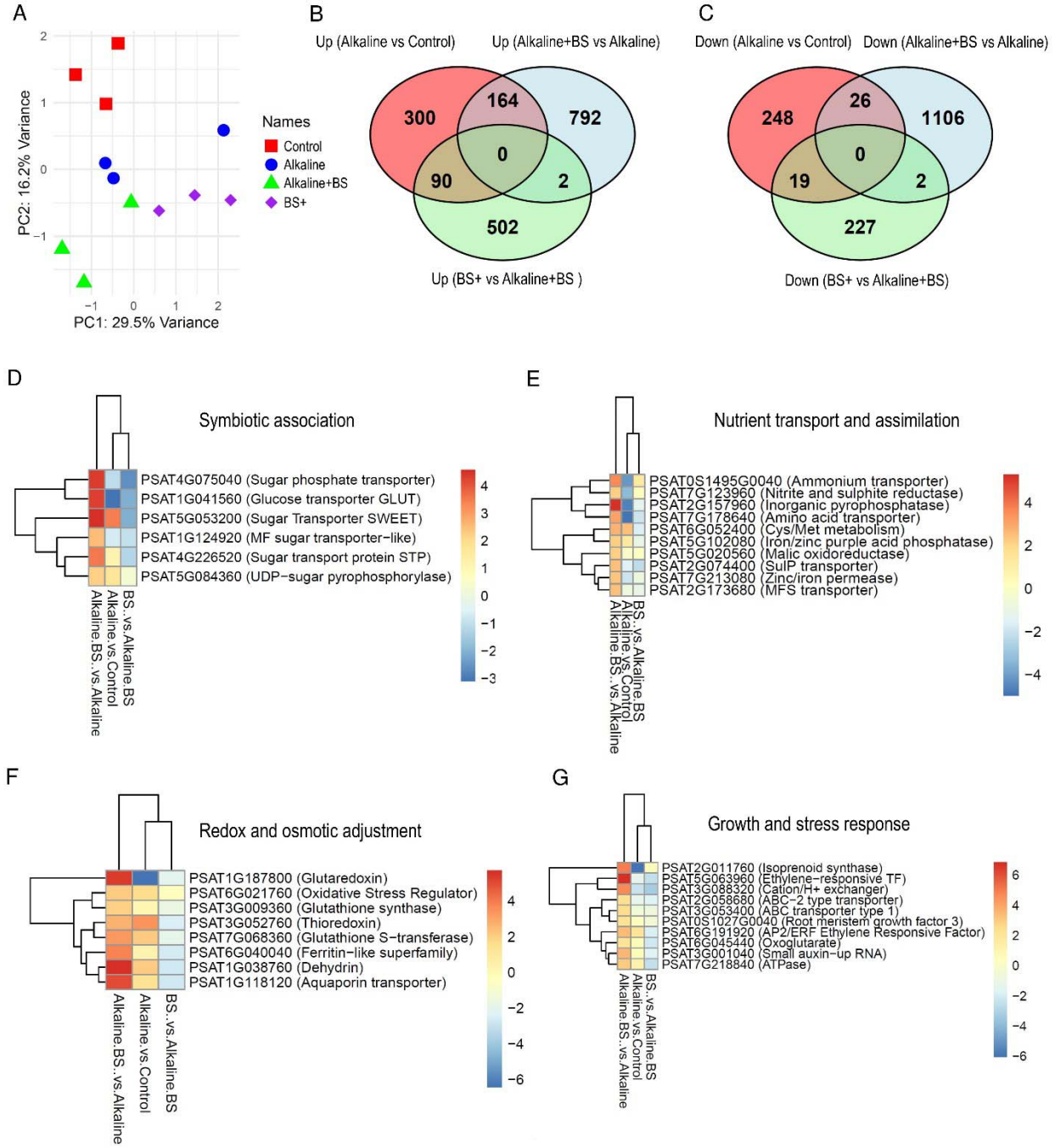

Fig. 5. Transcriptomic changes in pea roots under control, alkaline, alkaline + *B. subtilis* (alkaline+BS), and BS+ treatments: (A) Principal coordinate analysis (PCoA) plot showing separation of transcriptome profiles among treatments, (B) Venn diagram of upregulated genes, (C) Venn diagram of downregulated genes, (D–G) Heatmaps showing selected differentially expressed genes (DEGs) grouped by functional categories. Color scale represents normalized

expression values (log□ fold change), with red and blue indicating upregulation and downregulation, respectively. Data are based on three biological replicates per treatment (*n* = 3).

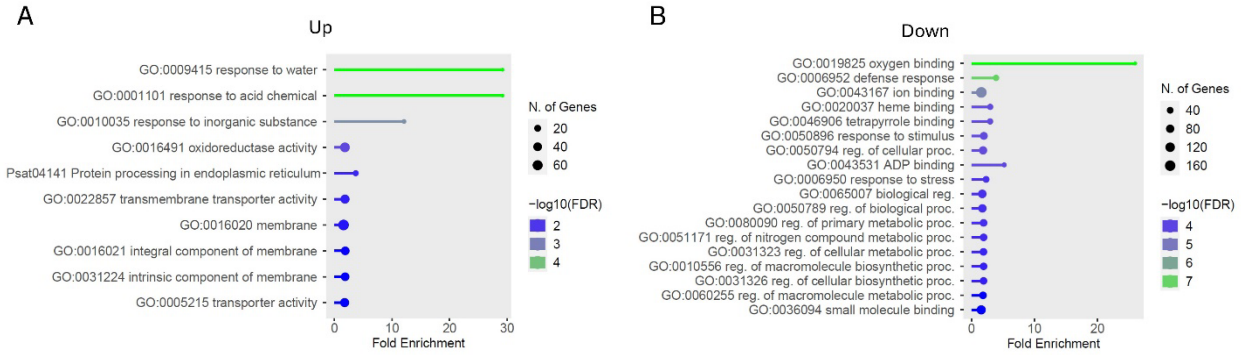

Fig. 6. Gene Ontology (GO) enrichment analysis of differentially expressed genes in the alkaline+BS *vs.* alkaline comparison using ShinyGO. (A) Top enriched GO terms for upregulated genes, including biological processes such as response to water, response to acid chemical, and response to inorganic substances, as well as molecular functions like oxidoreductase activity and transmembrane transporter activity. (B) Top enriched GO terms for downregulated genes, including oxygen binding, defense response, iron binding, and regulation of metabolic and biosynthetic processes. Bubble size represents the number of genes in each GO term, color indicates –log10(FDR), and the x-axis shows fold enrichment. Data are based on three biological replicates per treatment (*n* = 3).

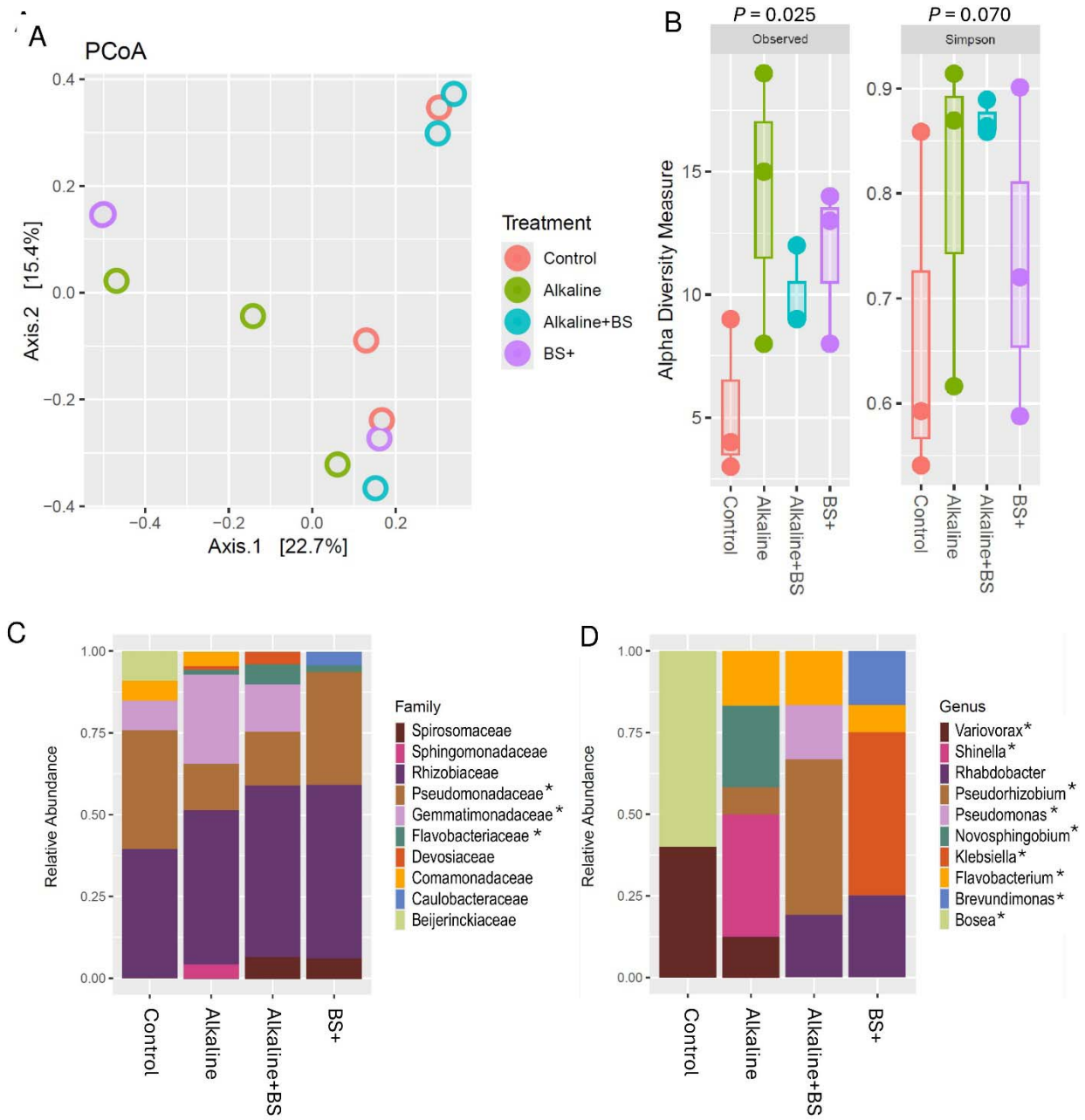

Fig. 7. Rhizosphere bacterial community structure in pea plants under different treatments: control, alkaline, alkaline+*B. subtilis* (BS), and BS+. Data represent (A) principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity showing separation of bacterial communities among treatments, (B) alpha diversity indices (Observed richness and Simpson index; box plots show median, interquartile range, and individual replicates, (C) relative abundance of dominant bacterial families, and (D) relative abundance of dominant bacterial genera. Asterisks (*) indicate taxa that showed statistically significant differences among treatments based on Kruskal-Wallis ($P < 0.05$). Data are from three biological replicates per treatment ($n = 3$).

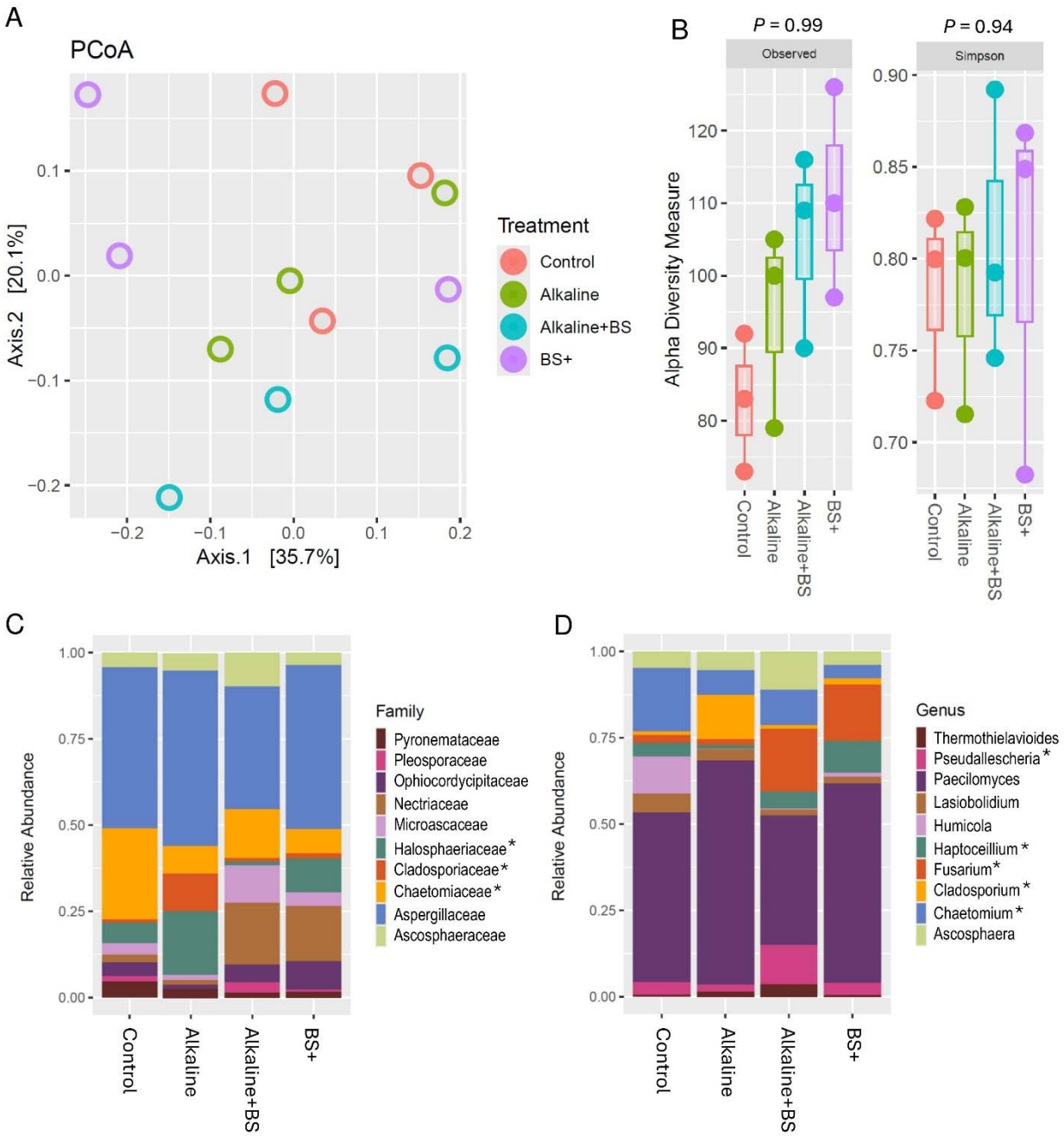

Fig. 8. Rhizosphere fungal community structure in pea plants under different treatments: control, alkaline, alkaline+*B. subtilis* (BS), and BS+. Data represent (A) principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity showing separation of bacterial communities among treatments, (B) alpha diversity indices (Observed richness and Simpson index); box plots show median, interquartile range, and individual replicates, (C) relative abundance of dominant fungal families, and (D) relative abundance of dominant fungal genera. Asterisks (*) indicate taxa that showed statistically significant differences among treatments based on Kruskal-Wallis ($P < 0.05$). Data are from three biological replicates per treatment ($n = 3$).

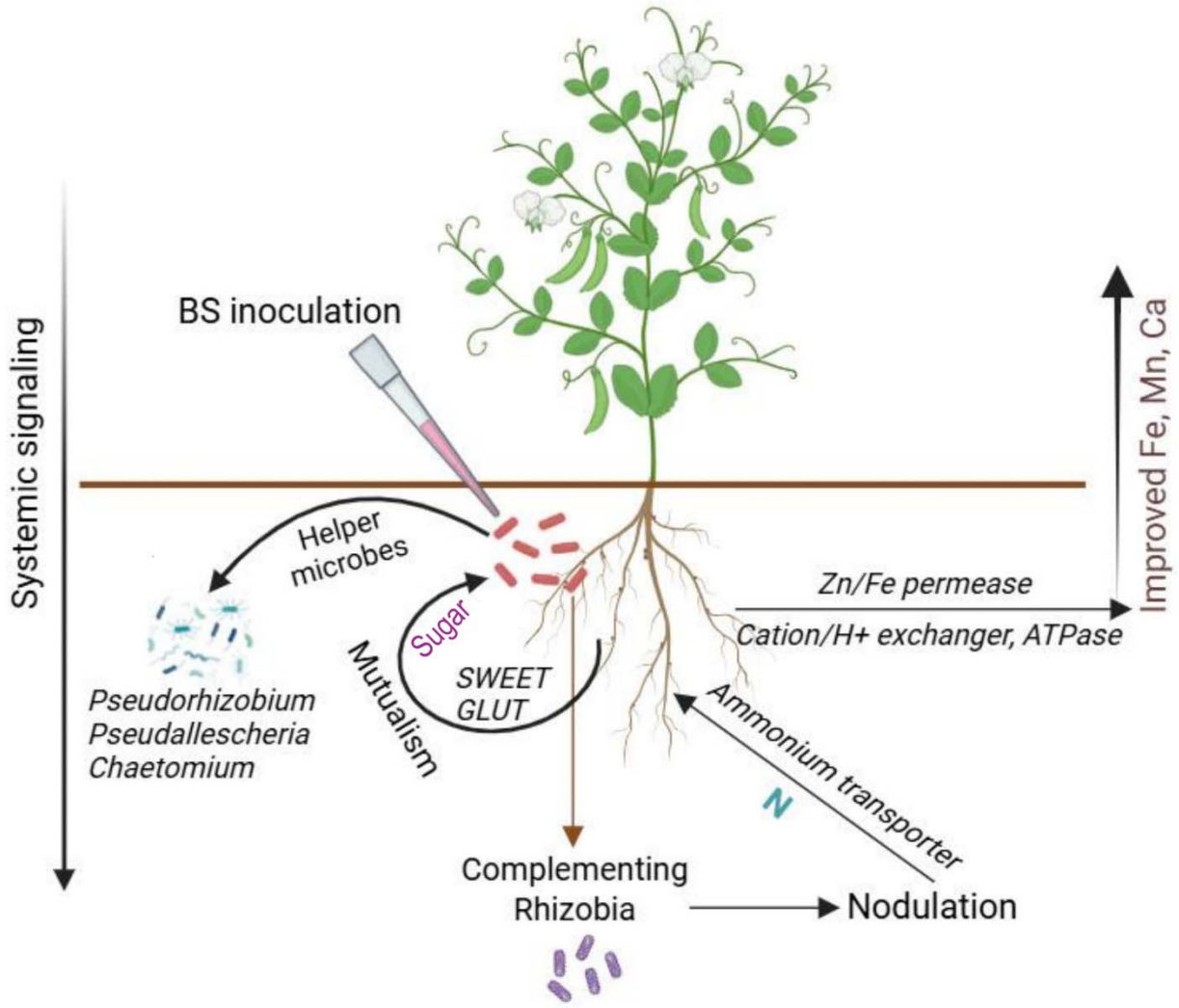

Fig. 9. Mechanistic model of *B. subtilis*-mediated strategies enhancing pea plant tolerance to alkaline stress. BS inoculation promotes colonization of helper microbes (*Pseudorhizobium*, *Pseudallescheria*, and *Chaetomium*), which establish mutualistic interactions with the host by exchanging sugars through SWEET and GLUT transporters. These interactions support complementing rhizobia, enhancing nodulation through ammonium transporters and nitrogen assimilation. In parallel, cation/H^+ exchangers, ATPases, and Zn/Fe permeases facilitate uptake of essential micronutrients (Fe, Mn, Ca). Together with systemic signaling, these mechanisms contribute to improved nutrient homeostasis and plant growth under alkaline conditions.