PLANT-MICROORGANISM INTERACTIONS

Imprints of PGPB association on the metabolic dynamism of *Piper nigrum*

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**ABSTRACT**

Endophytes are endosymbiotic microorganisms that coexist within different plant species which assist the host in multivariable ways without causing any detrimental effects on the plant well-being. The current study is focused on the bacterial isolates found in the *Piper nigrum* in vitro culture in the basal MS medium. The growth of these bacterial isolates even after repeated surface sterilization of the explant concludes the nature of these isolates as endophytes and these isolates were identified as Pantoea sp., Luteibacter sp., Herbaspirillum sp., and Agrobacterium sp. through 16SrRNA. The endophytes were tested for their potential to aid plant development by assessing the production of Indoleacetic Acid, Ammonia, Hydrogen Cyanide, 1-aminocyclopropane-1-carboxylic acid deaminase, Siderophore, fixation of Nitrogen, solubilization of Phosphate, heavy metal and salt tolerance. Pantoea sp. and Herbaspirillum sp. were found tolerant against salt and heavy metal stress respectively. Based on plant growth promotion assays, Pantoea sp. and Agrobacterium sp. were further selected for metabolomic profiling. The results indicated the effects of isolates on primary and secondary metabolite biogenesis, aminoacyl-tRNA synthesis and amino acid metabolic pathways. The profiling of important metabolites linked to crop development, revealing its metabolic mechanism of plant growth promoting activities facilitated through selected Plant Growth Promoting Bacteria.

1. Introduction

Plant beneficial microbes, which colonize rhizosphere and phyllosphere ensures better crop production through various interactions. Rhizospheric microbes colonize in the root zone where as phylospheric microbes colonize above-ground parts of plants (Afzal et al. 2019). Bacterial endophytes inhabiting the internal organs of the plants aid the host plant in varied ways via the release of plant growth promoters, exopolysaccharides, osmoprotectants enzymes (Berg et al. 2013), antifungal elements (Gond et al. 2015), and other useful compounds beneficial to the plants. It also assists in amending physiological and biochemical components and parameters of the plant (Beneduzi et al. 2012). Bacteria can enhance the germination of seeds and stimulate plant development by multitudes of processes such as the fixation of nitrogen, phosphate solubility, production of siderophore, and bioactive enzyme production (Liotti et al. 2018).

For the most part, rhizospheric and endophytic PGPB support plant growth through similar, but not identical ways. The fundamental difference is that once endophytic PGPB has established themselves within the host plant tissues, they are no longer affected by changing soil conditions. These circumstances which might impede the activity and development of rhizosphere PGPB include changes in temperature, soil pH, water content, and the existence of soil bacteria that compete on host-plants root surfaces for binding sites (Glick 2012). Endophytic bacterial diversity is generally more in root than the shoot tissue (Zinniel et al. 2002).

Detecting the presence of endophytes in in vitro plant tissue culture is an area that has received little consideration. In vitro culture can be considered as a helpful approach and a reliable source for recovering worthwhile bacteria found in certain organs (Moraes et al. 2012). Since endophytes only live inside the plant, it is critical to distinguish and eradicate any surface contaminants of plants to recover endophytes from plant tissues successfully. Plant materials are extensively surface sterilized in in vitro explant culture, yet surface sterilization procedures cannot prevent the growth of interior bacterial flora of plant tissues (Liaqat and Eltem 2016). The endophytic microbiome controls the production of varied metabolites in the endosphere and biotransforms specific plant-produced metabolites and stimulates the production of other components (Mahmood and Kataoka 2020). Metabolomic approaches can assist us in excluding the genomic data required for transcriptomic analysis. Furthermore, the cost of this analysis is less than that of transcriptomic profiling, making the metabolomics profile cost-effective (Munir et al. 2020).

Heavy metal contaminant has confirmed a significant environmental issue across the world. Plants that are exposed to high levels of heavy metal yield superoxide radicals, lead to oxidative stress (Efe 2020). Plants used in phytoremediation from associations with rhizospheric microorganisms in order to withstand heavy metal toxicity. Plant growth-promoting rhizobacteria (PGPRs) are a diverse group of bacterial genera that colonize the rhizosphere and thus play a role to plant adaptation to environmental circumstances (Rolón-Cárdenas et al. 2021).

In the current work, endophytic bacterial growth was observed in the in vitro cultures of *Piper nigrum*. These
isolates were identified and investigated for several plant growth-promoting characters and metabolic changes in *P. nigrum*. The purpose of the study was to better understand the cross-functional role of the phyllosphere endophytes in the rhizosphere to improve secondary metabolites in pepper plants.

2. Materials and methods

2.1. Plant materials

*Piper nigrum* L. var. Panniyur-1 was obtained from the College of Agriculture, Vellayani, Thiruvananthapuram, maintained in the pots at the Rajiv Gandhi Centre for Biotechnology greenhouse and used as experimental material for the study. Stem explants collected from the field were carefully washed with 2% (v/v) detergent solution 'teepol' and running tap water for one hour and swabbed with cotton wool dipped in 70% alcohol. Clean explant was treated with 0.3% bavistin (fungicide) followed by 0.1% (w/v) aqueous mercuric chloride solution. After each treatment, the explants were carefully cleaned with sterile water and allow the water to drain. The surface-sterilized explants were trimmed to 0.5–1 cm in length, comprising a single node with an axillary meristem or a shoot tip with an apical meristem and 1.0–1.5 cm internodal region. Half strength Murashige and Skoog salt medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar with 2.5 µM of benzyladenine (BA) per liter were used to obtain shoot cultures. The medium was adjusted to pH 5.7. Cultures were incubated at 27°C under 50–60 LEm⁻² s⁻¹ light intensity at 16-h light/8-h dark photoperiod. The plants were grown in the same medium. After two weeks, bacterial growth was observed in culture bottles. The explants were growing well even in the presence of bacterial growth. After four weeks, the explant was subcultured and transferred to fresh medium where the same bacterial growth was observed.

2.2. Isolation and characterization of endophytic bacteria

Bacterial cultures from in vitro *P. nigrum* were purified by streaking in Luria–Bertani (LB) agar medium and single colonies were obtained. Pure cultures were then grown in LB broth overnight at 37°C and after mixing with equal amount 50% (v/v) glycerol stored in – 80°C deep freezer in screw top tube after snap freezing with liquid nitrogen.

2.3. 16s rRNA sequencing

Genomic DNA was isolated from overnight grown cultures at 37°C using HiPurA Bacterial Genomic DNA Purification kit (HiMedia) following the manufacturer’s instructions and genomic DNA was amplified using Thermo Scientific PCR Master Mix and 1-27F (AGAGTTTGATCCTGGCTCA G) and 1495R (CTACGGCTACCTGTTACGA) primers for 16sRNA gene (Lane 1991). Agarose gel electrophoresis revealed the size of the fragment as 1500 bp. BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) was used for Sequencing PCR followed by sequencing using capillary electrophoresis. The sequencing data were analyzed using BLAST and MEGA 7, employing the Neighbor-joining technique for phylogenetic analysis (Tamura et al. 2013).

2.4. Screening of endophytic bacteria for plant growth promoting properties

2.4.1. Siderophore production

Samples were tested for siderophores production using the Chrome Azur S and HDTMA as indicators on Blue Agar–CAS Blue Agar medium (Schwyn and Neilands 1987). In order to produce the Blue Agar–CAS medium, we used autoclaved MM9 salt medium [added with 32.24 g piperazine-N, N0-bis2-ethanesulfonic acid, and 2.0 g citric acid with trace elements: 1 mg FeSO4·7H2O, 10 mg H3BO3, 11.19 mg MnSO4·H2O, 124.6 mg ZnSO4·7H2O, 78.22 mg CuSO4·5H2O, 10 mg MoO3, pH 7.2] amended with 3 mM ACC instead of (NH4)2SO4 as sole nitrogen source (Penrose and Glick 2003). The cultured plates were incubated at 28°C for 24 h before being analyzed. The appearance of a yellowish orange halo surrounding the colonies was interpreted as a sign of siderophores formation.

2.4.2. Phosphate solubilization

The phosphate solubilization ability by endophytic bacteria was tested for using the method described by Surange et al. (1997). Pikovskaya media containing (glucose 10 g/L, Ca3(PO4)2 5 g/L, (NH4)2SO4 0.5 g/L, NaCl 0.2 g/L, MgSO4·7H2O 0.1 g/L, KCl 0.2 g/L, Fe3O4·7H2O 0.002 g/L, yeast extract 0.5 g/L, MnSO4·H2O 0.002 g/L, agar 20 g/L, pH 7.2) and 2.4 mg/mL bromophenol blue was added to the medium. The cultures were seeded and incubated for 48 hours. After incubation, a yellow zone was spotted surrounding the colony due to the utilization of tricalcium phosphate present in the medium.

2.4.3. ACC deaminase production

Isolated bacterial cultures were grown in Luria–Bertani (LB) agar medium, and the plate was incubated for 24 h at 37°C. The different cultures were screened for ACC deaminase activity on the sterile minimal DF (Dworkin and Foster 1958) salts media (DF salts per liter: 4.0 g KH2PO4, 6.0 g Na2HPO4, 0.2 g MgSO4·7H2O, 2.0 g glucose, 2.0 g gluconic acid, and 2.0 g citric acid with trace elements: 1 mg FeSO4·7H2O, 10 mg H3BO3, 11.19 mg MnSO4·H2O, 124.6 mg ZnSO4·7H2O, 78.22 mg CuSO4·5H2O, 10 mg MoO3, pH 7.2) amended with 3 mM ACC instead of (NH4)2SO4 as sole nitrogen source (Penrose and Glick 2003). The cultured plates were incubated at 28°C for three days, and growth was observed on a daily basis. Colonies growing on the plates were indicated as ACC deaminase producers.

2.4.4. Screening of nitrogen fixation

Jensen’s medium was used to test bacterial isolates for preliminary screening of nitrogen fixation. The media contain (Sucrose 20 g/L, K2HPO4 1 g/L, MgSO4 0.5 g/L, NaCl 0.5 g/L, FeSO4 0.1 g/L, Na3MoO4 0.005 g/L, CaCO3 2 g/L), and agar 15 g/L was added to the media (Jensen 1942). Bacterial colonies were imprinted onto the medium and maintained for four days before being examined for growth (Jimtha et al. 2014). Further validation of nitrogen fixation potential was carried out by PCR amplification of a targeted region within the nitrogenase iron protein (nifH) gene. A nifH gene fragment (360 bp) was amplified by using two universal primers: (PoFr – TCGGAYCCSAARGCBGACTC and PoR – ATS GCCATCATYTTCRCGGGA) originally designed by Poly et al. (2001). Gel electrophoresis was used to analyze
the amplified products, as described earlier (Zhang and Chen 2012).

2.4.5. Ammonia production
The ammonia-producing ability of the endophytic isolates was tested. For this, the isolates were cultured into peptone water (Peptic digest 10 g/L, NaCl 5 g/L, dH2O 1000 ml, pH 7.2) and maintained for two days at room temperature before being screened for pathogenicity. After incubation, Nessler’s reagent (K2HgI4 and NaOH or KOH) was applied to the culture. The appearance of a brownish color indicated a positive outcome. Positive results (Cappuccino and Sherman 1992). Uninoculated cultures were inoculated onto LB agar with a glycine concentration of 4.4 g/L (Wei et al. 1991). Inside the lid of the culture plate, a Whatman no. 1 filter paper soaked in 0.5% of picric acid in 2% of Na2CO3 was placed. Plates were sealed and incubated for four days at 30°C. The shift in color changes of the filter paper from yellow to red was regarded as a positive outcome. As a negative control, uninoculated growth media was utilized (Millar and Higgins 1970).

2.4.6. Hydrogen cyanide production
Bacterial cultures were inoculated onto LB agar with a glycine concentration of 4.4 g/L (Wei et al. 1991). Inside the lid of the culture plate, a Whatman no. 1 filter paper soaked in 0.5% of picric acid in 2% of Na2CO3 was placed. Plates were sealed and incubated for four days at 30°C. The shift in color changes of the filter paper from yellow to red was regarded as a positive outcome. As a negative control, uninoculated growth media was utilized (Millar and Higgins 1970).

2.4.7. Indole acetic acid (IAA)
Endophytic bacterial isolates were inoculated into 10 ml of nutrient broth added with 0.2% (v/v) L-tryptophan and incubated at 28°C for 10 days. Following incubation, the culture was centrifuged at 3000 rpm for 20 min, and the supernatant was tested for the presence of Indole acetic acid (Rahman et al. 2010). One milliliter of culture supernatant was mixed with 2 ml of Salkowski reagent (35% HClO4, 0.5 M FeCl3) and incubated in tubes for 30 minutes in the dark. The appearance of red color was observed as an indication of a positive outcome. As a negative control, uninoculated growth medium was used.

2.5. In vitro assay for salt tolerance
Characterization of the isolates based on their performance in different concentrations of KCl and NaCl was carried out following Sharma et al. (2021). The LB agar medium amended with different concentrations of KCl and NaCl (5, 7.5, and 10%) were streaked with the bacterial isolates and incubated at 37°C for five days and growth was observed.

2.6. Heavy metal tolerance
Heavy metals tolerance assay was carried out by following method by Panchami et al. (2020) ZnCl2, CdCl2, and CoCl2 were added to LB medium to a final concentration of 100, 200, 400, and 800 ppm. The cultures were streaked on to LB plates and incubated at 37°C for days and growth was observed.

2.7. Effect of endophytic bacteria on growth promotion of P. nigrum plants
Piper nigrum L. seeds were collected from mature, healthy plants and were planted after sterilization on sterile soil in controlled environment. The three to four weeks old seedlings were used as a source of contamination-free plants for further experiments. The pots were filled with sterile soil and the healthy plants were planted (Single plant in each pot, pot size 5 × 5 × 4 inches, 1 kg soil). Twenty milliliters of overnight broth cultures (1 × 10^9 CFU mL⁻¹) of the isolates were inoculated to the 15 pepper seedlings including four treatments and one control three replicates each and morphological changes (up to 30 and 60 days) were observed in comparison with uninoculated control plants. The experiment was conducted twice.

2.8. Plant metabolomics
2.8.1. Bacteria inoculation and sample collection
Piper nigrum L. plants maintained at sterile conditions were inoculated with Pantoea sp. and Agrobacterium sp. Six plants were inoculated for both treatments (three replicates × two treatments) and three plants were maintained as control. The size of the inoculum was used 1 × 10^9 CFU mL⁻¹ each culture. After inoculation and incubation for 50 days, root and shoot samples were collected and stored in −80°C after snap freezing in liquid nitrogen until further use.

2.8.2. Metabolite extraction for GC-MS/MS
One gram of the shoot and root samples from control and treated plants were ground with liquid nitrogen in mortar and pestle. All experiments were conducted with three biological replicates and three technical replicates for each biological replicates. One hundred milligrams of sample from each replicates were mixed in 1 ml of chloroform/methanol/water ratio of 1:3:1 (v/v/v) and used for metabolite extraction. This mixture is then shaken for 30 min at 100 rpm in a 4°C shaker followed by centrifugation at 6000 g for 1 min at 4°C. The supernatant was collected and dried in a vacuum concentrator without heating.

2.8.3. Metabolomic statistical analysis
Statistical analysis was performed on obtained data after normalizing to % total ion count and log10-transformed in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca accessed on 8 February 2021). Differences in the metabolomic profiles of samples were analyzed with unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA). Significant variables were defined based on cross-validated p-values derived from one-way analysis of variance (ANOVA) with Bonferroni correction for false discovery rates (FDR). Multiple comparisons and post hoc analyses used Tukey’s Honestly Significant Difference (Tukey’s HSD). Fisher LSD test was used to determine which compounds varied significantly between groups at p < .05. Results were visualized in the form of hierarchical cluster analyses incorporating heat maps. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database mapped all significant metabolites into metabolomic pathways.

2.9. Development of GFP-tagged isolates
The selected bacterial isolates Pantoea sp. and Agrobacterium sp. were grown overnight at 37°C in 5 mL Luria–Bertani (LB) broth. This culture then inoculated to 50 ml LB broth in 250 ml Erlenmeyer flask till OD 600 = 0.5 ± 0.1 and the cells were harvested by centrifugation at 4°C, the pellet washed four or five times with 10% glycerol at 4°C, and the pellet resuspended using cold ultrapure 10% glycerol and electrophoresed (2.5 kV; 25 mA; 25 mF and 400 Ω).
with 0.1 μg of pBENGFP plasmid. After transformation, 1 mL of LB medium was added, incubated for 2 h at 37°C, and plated on LB medium supplemented with kanamycin (50 mg/ml). Colonies in the plates with antibiotic considered to have the plasmid. Fluorescence microscopy was also used to identify clones that contained the GFP.

2.10. Plant growth inoculation and confocal microscopic observation
Pepper seedlings growing on sterile conditions were used for inoculation with transformed isolates and observed under laser confocal (Olympus Fluoview™ FV1000 confocal microscope). The resulting images were obtained using the Cell science Viewer software or Zen 2012 software.

2.11. In silico analysis
Among the four isolates, Pantoea sp. showed more identity than other species submitted in NCBI (Pantoea dispersa 97.93, Accession number MT367789.1). The complete sequence of the species P. dispersa genomic sequence was retrieved on the website of NCBI (https://www.ncbi.nlm.nih.gov). The genome annotation assessment for PGP characteristics was carried out using the web software Rapid Annotation using Subsystem Technology (RAST). Furthermore, SEED user methods from the SEED website were employed to do a functional genomic analysis. The data analysis and genomic annotation for the P. dispersa help to clarify its unusual taxonomic categorization and its suitability for significant genome investigations. Overall, the genome provides information to validate, identify, and understand many of its previously studied properties crucial to promoting plant growth under varied stress circumstances (Chaudhary et al. 2021).

3. Results
3.1. Isolation of bacterial endophytes from P. nigrum
The surface-sterilized stem portion of P. nigrum inoculated in MS medium resulted in the isolation of four morphologically distinct entophytic bacterial isolates (Supplementary Figure S1). The obtained isolates were designated as CITDB to C4TDB and their different growth promoting competence described below. The bacterial cultures were purified by repeated streaking. The pure cultures were sequenced using 16S rRNA, and the sequences similarities were assessed with BLAST analysis. The pure cultures were stored in glycerol stock at −80°C for future uses.

3.2. Molecular identification
BLASTn algorithm was used to evaluate by the 16S rRNA sequences showed the similarity with other bacterial species in NCBI. Phylogenetic trees have been formed using a fragment of the 16S rRNA gene sequences to further validate the authenticity of the isolated strains to the genus level revealed above (Figure 1(A–D)). The sequence analysis of the 16S rRNA gene identified the isolates as Pantoea sp., Luteibacter sp., Herbaspirillum sp., and Agrobacterium sp. The BLAST search results indicate that none of the isolates remained close adequate to any reference strain sequence in NCBI, so we were incapable to identify the species level. However, the Pantoea sp. showed the similarity with P. dispersa 97.93% identity, NCBI Accession number MT367789.1.

3.3. Isolate, characterization as potential PGPB
3.3.1. Determination of siderophore, HCN, ammonia production, and phosphate solubilization
Chromazurol sulfonate agar plate method was used to examine the siderophore-producing capability of isolated PGPB from P. nigrum. The siderophore production was determined by the clear orange halo zone formed by the inoculated bacterial isolates on the chrome azurol S (CAS) agar media (Supplementary Figure S2(A)). All the four strains (Luteibacter sp., Pantoea sp., Herbaspirillum sp., and Agrobacterium sp.) were able to produce siderophore, Pantoea sp., and Luteibacter sp., showed higher siderophore production compared to other isolates. The HCN (hydrogen cyanide) production was examined in all the isolates, while the yellow picrate filter paper did not turn red-brown, indicating as isolated bacterial cultures did not produce HCN. Additional characteristic features of the PGP mechanism, Ammonia (NH3) production, were confirmed for the four isolates. All the cultures were positive for synthesis of ammonia in peptone water.

All four isolates (Luteibacter sp., Pantoea sp., Herbaspirillum sp., and Agrobacterium sp.) isolated from P. nigrum showed significant phosphate solubilization ability to alter inorganic forms of phosphorous TCP into the soluble form. The clear zone was formed around the colonies on Pikovskya’s a agar supplemented with 2% TCP (Supplementary Figure S2(B)), indicating the phosphate solubilization capability of the bacterial strains. The maximum solubilization index was shown by isolates Pantoea sp. and Herbaspirillum sp.

3.3.2. Indole acetic acid (IAA) and nitrogen fixation
Qualitative assay was used to detect the production of Indole acetic acid (IAA) in the isolated cultures. The cultures were able to produce IAA shown in the culture supernatant color converted from yellow to pink and to the appearance of pink color on and around the roots through a qualitative test shown in Supplementary Figure S3. Compared to other isolates, the sample Agrobacterium sp. showed higher IAA production ability. The preliminary screening of nitrogen fixation of the bacterial isolates was scrutinized by its ability to grow on nitrogen-free minimal medium (NFM). All the bacterial cultures were inoculated in this assay on nitrogen-free medium (NFM) and medium supplemented with 5 mM NH₄Cl used as a positive control. Our results indicated that all the cultures were able to grow on both plates, supplemented with or without nitrogen source. The ability of the isolates to fix nitrogen was confirmed by PCR amplification of a 360 bp DNA fragment in the nifH gene (Supplementary Figure S4). These results confirmed the N₂-fixation ability of the Pantoea sp., Luteibacter sp., Herbaspirillum sp., and Agrobacterium sp.

3.4. Evaluation of PGPBs to plant growth promotion of P. nigrum
The preliminary PGP test was done using one-month-old pepper seedlings (Supplementary Figure S5). The isolated cultures of Pantoea sp. and Agrobacterium sp. showed higher plant growth promoting ability compared to other cultures.
Based on the above-mentioned PGP assays finally, *Pantoea* sp. and *Agrobacterium* sp. cultures were selected for greenhouse assay and metabolomic profiling of *P. nigrum*. The differences within the inoculated plants were measured and recorded. The plant-growth-promoting (PGP) and metabolomics effects of the cultures were evident after 50Dai (Day After Inoculation) in a greenhouse. The *Pantoea* sp. and *Agrobacterium* sp. significantly increased the length of the roots, shoots, and leaf number of entire *P. nigrum* plants compared to the uninoculated control (Figure 2).

### 3.5. Screening of bacteria isolates for salt tolerance

Furthermore, all the four isolates were tested in vitro for their ability to withstand high salt concentrations (5, 7.5, and 10%) of NaCl and KCl. In terms of salt tolerance, the isolate *Pantoea* sp. and *Luteibacter* sp. grew on LB medium supplemented with 5% NaCl, whereas the *Pantoea* sp. grew on LB medium supplemented with 7.5% NaCl. No isolates were unable to resist the addition of a high quantity of NaCl (10%) to the growth media. At the same time, *Pantoea* sp. and *Luteibacter* sp. grow on high KCl supplemented with 5 and 7.5%, and the *Pantoea* sp. were grown on 10% of KCl (Supplementary Figure S6).

### 3.6. Screening of heavy metal resistant potential

To examine the growth in heavy metal (cobalt, cadmium, and zinc) contain (100 ppm to 800 ppm) LB medium, the isolates were inoculated after two days of incubation exposed that each of the bacterial isolates contains heavy metal decay-potential, as they can grow thereby degrading heavy metals. In all the four isolates *Herbaspirillum* sp. and *Agrobacterium* sp. showed metal tolerance in 800 ppm followed by *Pantoea* sp. and *Luteibacter* sp. were grown in zinc and cobalt containing LB medium up to 400 ppm. In cadmium, the isolates *Herbaspirillum* sp. and *Agrobacterium* sp. were grown up to 400 ppm compared to other isolates. In higher concentration of cadmium 800 ppm, the growth was retard (Supplementary Figure S7).

### 3.7. Colonization of black pepper roots by GFP-labeled *Pantoea* and *Agrobacterium*

In order to generate isolates strains treated with GFP for plant interaction imaging experiments, the plasmid pBENGFP was transformed into the endophytic *Pantoea* and *Agrobacterium* strain by electroporation. The GFP-transferred bacterial cells were grown overnight at 37°C and inoculated black pepper roots. After five days of inoculation, root samples were analyzing the fluorescence to examine the colonization potential of selected bacterial strains in the root (Figure 3).

### 3.8. Untargeted metabolic profiling of PGPB-induced changes in black pepper tissues

After 50 days post-inoculation, untargeted metabolomics profiling was used to examine metabolic changes in black pepper shoot and root treated with the two isolates *Pantoea* sp. and *Agrobacterium* sp. The experimental setup has comprised of three independent biological repeats, and the samples were examined in triplicate. These PCA-selected metabolic features were validated and complemented by orthogonal projection to latent structures discriminant analysis (OPLS-DA) modeling. The supervised clustering method, Partial Least Squares-Discriminant Analysis (PLS-DA), was used to shoots and roots treated with inoculate and compared with control. Using the five-component concept, the PLS-DA method identified important metabolic pathways depending on the VIP score. PLS-components (PCs) scrutiny of shoots found that module 1 defined 17.6% and 29.8% of the overall difference between the two treatments against the control, respectively. The root treated with PGPB and the uninoculated control was subjected to the PLS-DA analysis. The first PLS constituent (PC1) elucidated 28.5% and 19.3% of the alterations across the treatment and control roots data sets, respectively (Supplementary Figure S8(A,B)). This distinction between treated and untreated samples demonstrates how PGPB affects the state of metabolites in black pepper.

The metabolites of both the PGPB (*Pantoea* sp. and *Agrobacterium* sp.) treated and control shoot were grouped into two main clusters (Supplementary Figure S9(A)). The majority of metabolites are divided into two subclusters in Cluster-1. Subcluster-1 contains the majority of control shoot metabolites, whereas subcluster-2 contains *Agrobacterium* sp.-treated shoot metabolites. Cluster-2 shows the shoot metabolites of *Pantoea* sp. treated samples. In the case of root dendrogram analysis, metabolites share of both the PGPB treated and control were distributed into two main clusters (Supplementary Figure S9(B)). Cluster 1 has the
treated root metabolites with two bacteria and cluster 2 has control root metabolites.

### 3.9. Heat map

Amino acids, vitamins, as well as other substances were considerably accumulated in the shoot and root of plants treated with PGPB, among another clusters of metabolites. Hierarchical cluster pattern combined with a heat map was used to visualize the significance changes of PGPB-treated plants on metabolomics fold changes versus inoculated and uninoculated control plants. The heat plot was created by comparing treated shoot and root samples to control samples. The heat map represents the relationship among control metabolites.
and that the metabolites of the two treatments are combined together. D-Glucuronic acid, Citric acid, Tyramine, Dopamine, L-Asparagine, Ethanolamine, L-Threonine, Benzoic acid, Propanedioic acid, Palmitic acid, and L-The abundance of phenylalanine was higher in pepper shoots treated with Pantoea sp.

In contrast, Heptasiloxane, Benzeneacetic acid, D-Lactose, D-Fructose, Xylose, hexos, Pyrogallol, Sinapyl alcohol, Hydracrylic acid, D-Glucitol, D-Galactose, and Lactose showed higher expression in Agrobacterium treated samples correlated to control plants (Figure 4). The heat plot of roots showed the variation of metabolic changes in treated plants with bacterial isolates compared to control samples. Turaose, Hydroxybytric, Quininic acid, Dihydroxyphenyl Glycine, Ethanolamine, L-Rhamnopyranose, Pentanedioic acid, aminobutanoic acid showed higher accumulation in Pantoea treated roots of pepper. In the case of Agrobacterium treated roots, the compounds such as Tyramine, Propanediol, D-Fructose, D-Glucose, Maltose, Malto, Galacturonic acid, Shikimic acid, Glycolic acid, Allose, Dulcitol, Adonitol, Cellobiose, Silanol, Glucitol, and Lactic Acid were showed higher accumulation compared to control plants (Figure 5). Pantoea showed higher metabolism changes in pepper shoots compared to root and in the case of Agrobacterium, it showed metabolic changes in root samples of pepper plants.

The cell color and concentration depict metabolic regulation (up/down) in responding to PGPB inoculum. Significantly increased metabolites are shown in red, whereas substantially reduced metabolites are shown in blue. Mainly two patterns can be seen on both sides (top and left), which are further separated into different clusters. Such clusters depict the correlation between both the circumstance and the identified metabolomic content/concentration through response to PGPB strain inoculum.

### 3.10. Metabolic pathway analysis

Arabidopsis thaliana was used as the pathway library to better understand the fundamental activities of discovered shoot metabolites. Table 1 represents the shoot metabolites directly implicated in every pathway, as well as the number of hit metabolites and the pathway’s FDR. As predictable, such metabolites were discovered to be associated in a variety of pathways. MetaboAnalyst5 was able to determine metabolites present in biosynthetic pathway, including Aminoacyl-tRNA biosynthesis, Glyoxylic acid, and dicarboxylate metabolism, Valine, leucine, and isoleucine biosynthesis, Phenylalanine, tyrosine and tryptophan biosynthesis, Lysine biosynthesis, Glucosinolate biosynthesis, Indole alkaldoid biosynthesis, Amino sugar, and nucleotide sugar metabolism, and pyridine alkaloid biosynthesis. In the root, the metabolites are involved in each pathway, namely Galactose metabolism, Pentose and glucuronate interconversions, Biosynthesis of unsaturated fatty acids, Isoquinoine line alkaloid biosynthesis, Glycolysis/Gluconeogenesis, Amino sugar and nucleotide sugar metabolism, Glyoxylate, and dicarboxylate metabolism, Fatty acid degradation, Tyrosine metabolism, Butanoate metabolism, Ascorbate, and alternate metabolism, Fatty acid elongation, Fatty acid biosynthesis, Glutathione metabolism, Inositol phosphate metabolism, Glycine, serine and threonine metabolism, Arginine and proline metabolism, Glycerophospholipid metabolism, and Aminoacyl-tRNA biosynthesis were also drastically impact on PGPB treatments (Table 2).

### 3.11. In silico analysis

The *P. dispersa* strain genome was assessed in order to learn much more about PGP traits and genes involved in different stress responses aspects. In particular, the annotated genome provides data to verify, distinguish, and comprehend most of those formerly evaluated characteristics futures of the organisms that are critical to plant growth development under different stress conditions. Sulfur, protein, and RNA metabolic activity; capsule as well as cell wall formation; potassium and nitrogen fixation; iron acquisition; defense, disease, and pathogenicity processes; membrane transport; stress reaction; aromatic component metabolic activity; cell signaling regulation; pigment regulation; and vitamin supplements and prosthetic group identity are just a few of the genes found in this species (Figure 6 and Table 3). According to this analysis, the genome of *P. dispersa* contained 26 genes involved in iron acquisition and metabolic activity. Those certain genes (18 in total) express for the production of siderophore, which aids in iron uptake. Furthermore, PGP characteristics confirmed traits like siderophore and ammonia production, which were discovered to aid in aerobactin biosynthesis and ammonia assimilation.

Furthermore, the genome of *P. dispersa* contains 150 stress-response genes, including 65 for oxidative stress, 19 for osmotic stress, cold shock 2, heat shock 16, and 23 for detoxification. Osmotic stress-related genes are involved in choline and betaine biosynthesis. Osmotic tolerance is also mediated by glutathione biosynthesis and the gamma-glutamyl cycle. In addition to the stress reactions, eight genes are responsible for pathogen defense mechanism. As a result, these genes code for a variety of stress-related mechanisms.

### 4. Discussion

Bacteria growing in tissue culture bottles with *P. nigrum* L. were identified as endophytes. To the best of our knowledge, there has been no information concerning isolation of endophytic bacteria from pepper grown in vitro. There were only a few research have been published on the isolation of endophytic microbes from micro propagated plants (de Almeida et al. 2009). Current study identified certain isolates from the in vitro cultures and was characterized.

These bacterial isolates were found harmless to the growing pepper on basal MS medium and it can be beneficial to the host as Plant Growth Promoting Bacteria (PGPB). These endophytes aid in plant growth by producing siderophores, IAA, ACC deaminase, solubilizing phosphates, and fixing nitrogen. IAA-producing endophytes can enhance the root growth in plants and improve nutrient uptake (Mendes et al. 2007; Li et al. 2008). All the isolates were able to produce IAA, the production potential vary among them because of the variations in synthetic pathways, essential genes, and regulatory strategies (Wang et al. 2020).

Phosphorus is one of the macronutrients essential for proper plant growth and development. Most of the phosphorus in soil is unavailable to the plants and PGPBs can make phosphorus available to the plants by solubilizing it and improves the plant growth. Applying these Phosphorus Solubilizing Microbes (PSM) to the soil can reduce the use of chemical phosphorus fertilizers (Kumari et al. 2018). All the four isolates in this study were recognized as PSMs.
Figure 4. Heat map analysis of untargeted shoot metabolites of *Piper nigrum* treated with *Pantoea* sp. and *Agrobacterium* sp. (A) Metabolic pathway. (B) Key pathways. (C) Shoot heat map (PS = Pantoea treated shoot, AS = Agrobacterium treated shoot, and CS = Control shoot). (D) Box plot of specific metabolites.

Figure 5. Heat map analysis of untargeted root metabolites of *Piper nigrum* treated with *Pantoea* sp. and *Agrobacterium* sp. (A) Metabolic pathway. (B) Key pathways. (C) Shoot heat map (PS = Pantoea treated root, AS = Agrobacterium treated root, and CS = Control shoot). (D) Box plot of specific metabolites.
Table 1. Pathway names, total metabolites involved in that shoot pathways, metabolites significantly accumulated in the present study (hits), and false discovery rate (FDR).

| Pathway name                        | Total | Hits | FDR          |
|------------------------------------|-------|------|--------------|
| Aminocyl-tRNA biosynthesis         | 46    | 8    | 0.023176     |
| Glyoxylate and dicarboxylate metabolism | 29   | 6    | 0.028365     |
| Valine, leucine, and isoleucine biosynthesis | 22  | 4    | 0.27379      |
| Galactose metabolism               | 27    | 4    | 0.36384      |
| Isoquinoline alkaloid biosynthesis | 6     | 2    | 0.36384      |
| Cyanocarnoic acid metabolism       | 29    | 4    | 0.36384      |
| Glycine, serine, and threonine metabolism | 33  | 4    | 0.41146      |
| Stilbenoid, diarylheptanoid, and gingerol biosynthesis | 8  | 2    | 0.41146      |
| Propanoate metabolism              | 20    | 3    | 0.41146      |
| Phenylalanine metabolism           | 11    | 2    | 0.61348      |
| Phenylpropanoid biosynthesis       | 46    | 4    | 0.74088      |
| Sulfur metabolism                  | 15    | 2    | 0.74088      |
| Alanine, aspartate, and glutamate metabolism | 20  | 2    | 0.74088      |
| Pentose and glucuronate interconversions | 16  | 2    | 0.74088      |
| Tyrosine metabolism                | 16    | 2    | 0.74088      |
| Beta-Alanine metabolism            | 18    | 2    | 0.84734      |
| Valine, leucine, and isoleucine degradation | 37  | 3    | 0.89122      |
| Citrate cycle (TCA cycle)          | 20    | 2    | 0.89759      |
| Alanine, aspartate, and glutamate metabolism | 22  | 2    | 0.89879      |
| Biosynthesis of unsaturated fatty acids | 22  | 2    | 0.89879      |
| Pantothenate and CoA biosynthesis  | 23    | 2    | 0.91962      |
| Glucosinolate biosynthesis         | 65    | 4    | 0.94888      |
| Glutathione metabolism             | 26    | 2    | 0.95584      |

Siderophores are group of molecules produced by certain microorganisms which helps in obtaining iron from the surroundings. This chelation of ferric iron makes it suitable biocontrol agent by inhibiting the growth of phyto-pathogens. This iron is also available for the plants as a nutrient (Chandra et al. 2021). All four isolates tested positive for this test. Like siderophores, ammonia produced by these PGPRs also inhibits the growth of pathogenic microbes by increasing the pH (Gupta and Pandey 2019). All of the isolates assayed in the current study were ammonia producers.

The ability of microorganisms to enhance crop growth while also being able to endure heavy metals is a well-known feature of PGPR. They can reduce heavy metal toxicity while also promoting plant growth and function. The selection of metal-tolerant microbes that are also efficient producers of PGP substances can help to accelerate colonization of the plant rhizosphere in contaminated soils. *Bacillus, Micrococcus, Arthrobacter, Sphingomonas,* and *Microbacterium* were among the most common metal-tolerant gram-negative and gram-positive bacteria (Panchani et al. 2020). In the present study, the bacterial isolates *Herbaspirillum* sp. and *Agrobacterium* sp. showed metal tolerance ability compared to other cultures Supplementary Figure S7.

The synergistic effects occur when two or more dependent bacterial cultures are grown together. Such synergism can be made use of in plant growth promotion as a feasible technique for increasing the activity and viability of plant-growth-promoting microorganisms (PGPM), as indicated in previous studies (Singh et al. 2014). Growth-enhancing compounds furnished by the two microorganisms that affected plant metabolic developments may be responsible for the improved black pepper growth parameters as is evident from the present study. Compared to the control, PGPB-treated plants had significantly higher root and shoot length and as well as biomass. Recently reported that the conjugative effect of PGPR and effective Tilak et al. (2006) *Rhizobium* strains influenced pigeon-pea development and nitrogen fixation by assisting in the occupancy of introduced *Rhizobium*.

Mhlongo et al. (2020) revealed metabolic variations in PGPR-treated and untreated control tomato plants using UHPLC-MS. Furthermore, amino acids, organic acids, HCA derivatives, hydroxybenzoic acids, fatty acids, and glycoalakoloids were involved in this metabolic reprogramming. In addition, differential changes in several phytohormones were also seen during drought stress. These molecules function as essential signaling chemicals in reaction to abiotic and biotic stressors. Phytohormones can operate synergistically or antagonistically to facilitate in fine-tuning trade-off of assets among the development and defensive responses in plants depending on the conditions prevailing (Nepali et al. 2021).

In the current study, L-asparagine levels enhanced in pepper plants inoculated with the PGPB. Asparagine is essential for plant growth and development. Accumulation of this in legumes has previously been shown to regulate nitrogen fixation under drought conditions as well as nitrogen transport. Higher asparagine accumulation could be a drought stress management strategy of PGPR-treated plants (Khan et al. 2019a, 2019b). Forde and Lea (2007) and Curtis et al. (2014), revealed an increased asparagine absorption in response to various biotic and abiotic stresses which concur with our results.

MetaboAnalyst software was used to the link among various metabolic pathways and related metabolites. The Kyoto Encyclopedia of Genes and Genomes database (KEGG) and the *Arabidopsis* annotated project data model considerably altered 25 metabolic pathways. The production of phenylalanine, tyrosine, and tryptophan was increased in PGR (plant growth regulators) and PGPR inoculated plants. This mechanism facilitates the production of important aromatic amino acids. Moreover, protein biosynthesis, such aromatic amino acids are associated with the production of many other essential secondary metabolites which are important for growth and development of plants (Khan et al. 2019a, 2019b). In our study, PGPB treatment also found to alter the aminocyl-tRNA biosynthesis and the citrate cycle. Aminocyl-tRNA biosynthesis involves a group of 20 enzymes that regulates genetic codes. It has

Table 2. Pathway names, total metabolites involved in that root pathways, metabolites significantly accumulated in the present study (hits), and false discovery rate (FDR).

| Pathway name                        | Total | Hits | FDR          |
|------------------------------------|-------|------|--------------|
| Galactose metabolism               | 27    | 3    | 1.00E+00     |
| Pentose and glucuronate inter conversions | 16  | 2    | 1.00E+00     |
| Biosynthesis of unsaturated fatty acids | 22  | 2    | 1.00E+00     |
| Isoquinoline alkaloid biosynthesis | 6     | 1    | 1.00E+00     |
| Glycolysis/glucconeogenesis         | 26    | 2    | 1.00E+00     |
| Amino sugar and nucleotide sugar metabolism | 50  | 3    | 1.00E+00     |
| Glyoxylate and dicarboxylate metabolism | 29  | 2    | 1.00E+00     |
| Fatty acid degradation              | 37    | 2    | 1.00E+00     |
| Tyrosine metabolism                | 16    | 1    | 1.00E+00     |
| Butanoate metabolism               | 17    | 1    | 1.00E+00     |
| Ascorbate and aldarate metabolism   | 18    | 1    | 1.00E+00     |
| Pentose phosphate pathway           | 19    | 1    | 1.00E+00     |
| Fructose and mannose metabolism    | 20    | 1    | 1.00E+00     |
| Citrate cycle (TCA cycle)          | 20    | 1    | 1.00E+00     |
| Glycerolipid metabolism            | 21    | 1    | 1.00E+00     |
| Pyruvate metabolism                | 22    | 1    | 1.00E+00     |
| Thiamine metabolism                | 22    | 1    | 1.00E+00     |
| Starch and sucrose metabolism      | 22    | 1    | 1.00E+00     |
| Phenylalanine, tyrosine, and tryptophan biosynthesis | 22  | 1    | 1.00E+00     |
| Alanine, aspartate, and glutamate metabolism | 22  | 1    | 1.00E+00     |
| Fatty acid elongation               | 23    | 1    | 1.00E+00     |
| Fatty acid biosynthesis             | 56    | 2    | 1.00E+00     |
previously been described that interrupted metabolic circumstances are associated with a certain aminoacyl-tRNA synthetase (Herrmann 1995). Aminoacyl-tRNA synthetases act as a catalyst in the adhesion of amino acids to their particular tRNA and, as a result, play a significant role in translation and gene expression. The citrate cycle yields ATP and fulfills the requirement of carbon skeletons for a variety of biosynthetic pathways (Akram 2014).

The metabolite groups of groundnut differ due to the influence of PGPB, including amino acids, carboxylic acids, and sugars, which are correlated using the ANOVA test (D-glucopyranoside, pentanoic acid, 2-pyrrolidinone, proline, valine, benzoic acid, propanedioic acid, tartaric acid, pentanedioic acid, and 5-methyluridine). These metabolites whose presence in PGPB inoculated treatments were found to enhance functional characteristics (Ankati and Podile 2019). Our results showed differential metabolic changes in treated plants compared to uninoculated control and it is possible that an endophytic bacterial response can undertake specialized roles that enhance plant growth.

Nitrogen is stored in plants majorly as arginine, which then transformed into amino acids as well as urea to be used by the plants (Mahmood and Kataoka 2020). In this study, the concentration of urea was relatively higher in plants treated with endophytic bacteria, which could be attributed to increased nitrogen accessibility to the plants assisted by bacteria. There have been few studies upon that chemoattractants of PGPR which are impacted by 2-methylbutyric acid, stearic acid, palmitic acid, and oleic acid. The presence of obstructive water condition heightened the efflux of palmitic, palmitoleic, stearic, and oleic acid in pea-nut REs, preferring PGPR mobility, chemotaxis, and root attachment (Xiong et al. 2020). In our findings stearic acid, palmitic acid concentration was higher in PGPB-treated pepper plants.

The concentrations of proline and oxoproline were higher in bacterial treatments and these amino acids are well known for their role in cell osmoregulation (Aguiar et al. 2018). Proline being an osmoprotectants plays critical role in stress resistance in plants. Inoculation with Pantoea sp. increased the proline content of P. nigrum shoot and root, according to our findings. This is consistent with recent findings that showed higher proline levels following PGPB treatment, which reduced the impact of salt stress on pepper and tomato seedling growth (Xiong et al. 2020). One of the most common environmental challenges to crop yield and quality is increased soil salinization. This issue has been identified as one of the significant barriers to crop productivity (Sharma et al. 2021). In the present study, four isolates were salt-tolerant at various salt concentrations. The isolate Pantoea sp. showed the most incredible tolerance to salts up to 10% whereas the others failed to survive beyond 5% (Supplementary Figure S6).

Table 3. Representation of different genes encoding for PGP traits and their roles.

| S. No. | Genes for PGP traits | Role |
|--------|----------------------|------|
| 1 | Nitrogen metabolism genes | Nitrogen regulation protein NR(I) | Glutamate-ammonia-ligase adenyllyltransferase |
| 2 | Nitrogen metabolism genes | NtrR | Nitrite-sensitive transcriptional repressor |
| 3 | Genes for defense mechanism | CzcD | Cobalt-zinc-cadmium resistance protein |
| 4 | Genes for defense mechanism | MFP | Membrane fusion component of tripartite multidrug resistance system |
| 5 | Stress responsive genes | CysA | Sulfate and thioulate import ATP-binding protein CysA |
| 6 | Stress responsive genes | GT | Glutathione S-transferase |
| 7 | Stress responsive genes | Redox | Redox-sensitive transcriptional regulator (AT-rich DNA-binding protein) |
| 8 | Stress responsive genes | ZUR | Zinc uptake regulation protein ZUR |
| 9 | Iron acquisition and metabolism genes | Feat | Iron-chelator utilization protein |
| 10 | Iron acquisition and metabolism genes | FhuA | Act as an outer membrane receptor |
| 11 | Iron acquisition and metabolism genes | FhuB | Act as an ABC transporter |
| 12 | Iron acquisition and metabolism genes | FhuD | Periplasmic substrate binding protein |
| 13 | Sulfur metabolism genes | DtpA | Dl/tripeptidepermease DtpA |
| 14 | Sulfur metabolism genes | DtpT | Dl/tripeptidepermease DtpT |
| 15 | Sulfur metabolism genes | TonB | Fferric siderophore transport system, periplasmic binding protein TonB |
| 16 | Sulfur metabolism genes | DsrE | Sulfite reduction-associated complex DsrMKJOP and co-clustering genes
Phenylpropanoid pathway acids such as cinnamic, ferulic, and chlorogenic acids were present in PGPB-treated plants and less quantity in controls, indicate that PGPBs induce production of these compounds. Antifungal activity of these compounds is well studied (Singh et al. 2003). Our results reveal the presence of cinnamic and chlorogenic acids in *Pantoea* treated shoot. According to Rudrappa et al. (2008), malic acid/malate is a metabolite that promotes plant – PGPR communication in roots and knockout *Arabidopsis* mutants for malic acid transporter were unable to recruit PGPR for symbiosis. The presence of malate in shoot and root of PGPB inoculated pepper plants facilitates colonization of PGPB.

Late in plant development, the chemotaxis family, a response regulator involved in bacterial chemotaxis, is significantly expressed, and its expression is positively correlated with root exudates glycine and xylitol. PGPRs and endophytic bacteria chemotactically respond to glycine, whereas the non-symbiotic nitrogen-fixing bacteria *Azotobacter vinelandii* chemotactically responds to xylitol (Chaparro et al. 2014). Current study also showed higher expression of xylitol in PGPB inoculated pepper.

Phenylpropanoids are phenylalanine derivatives with a skeleton of C6-C3 (phenyl-propane). The shikimate pathway also produces the essential amino acid phenylalanine, as well as the aromatic amino acids tyrosine and tryptophan. This pathway’s precursors are phosphoenolpyruvate, which comes from glycolysis, and erythrose 4-phosphate, which comes from pentose phosphate (Aguiar et al. 2018). The shikimic pathway was staggering changed by inoculation. The treatments increased the concentration of both compounds. Malic acid and fumaric acid are associated with drought adaptation, owing to the osmolytic properties, the ability to regulate intracellular ionic content and stomatal conductance. Increased levels of proline, serine, threonine, cysteine, alanine, aspartic acid, phenylalanine, and tyrosine have been linked to drought-mitigating mechanisms like stomatal regulation, osmotic adjustments, and oxidative stress protection (Nephal et al. 2021).

Previous research indicated that the genes *cysA*, *cysP*, and *cysW* aid in transporting thiosulfate and sulfates in PGPR-producing *Pseudomonas* species. Under stressful conditions such as low temperature, high salinity, and osmotic stress, the *otsA/otsB* pathway has also been studied. Furthermore, the various strains of PGPR code for copper resistivity via the *copA*, *copB*, and *copC* genes (Chaudhary et al. 2021). Overall, the annotated genome of *P. dispersa* retrieved from NCBI provides evidence to confirm, identify, and recognize many of the strain’s previously assessed different properties that are related and important to plant growth promotion under innumerable conditions.

The metabolomic study also confirmed that little is known about endosymbionts interactions with their hosts, as evidenced by the lack of even a single metabolite concentration similarity between the two treatments of shoots (Figure 4); this indicates that, while these two endophytes enhanced plant growth to similar degrees in pot experiments, there was a significant difference in the metabolites detected within the plant tissues. As a result, each endophytic bacterium plays a unique role in plant growth; thus, studying axenic PGP traits alone may not be sufficient for screening isolates; instead, actual plant growth experiments are required. Endophytic interactions with host plants are thus a difficult process, and endophytic bacteria can produce or induce the production of specific metabolites that help plants grow faster.

## 5. Conclusion

In the current work, we described the changes in the metabolite profile in *P. nigrum* inoculated with PGPB. However, we found some in vitro endophytes isolated from pepper could produce the growth regulator IAA, siderophore, fix nitrogen, and solubilize phosphate. Endophytic bacteria isolated from pepper promoted plant growth at various scales and regulated various metabolites within the host plant’s endosphere. Endophytic bacterial strains that possess certain PGP traits and enhance plant growth without chemical fertilizers provide insight into their use for sustainable crop production. The underlying mechanisms may differ because different bacteria contributed different amounts to enhancing plant growth or metabolite release. To better understand the role of applied microbes, future research should look into the metabolites produced by microbes and plants separately. Similarly, metabolomic comparisons between endophytic effects in rhizosphere could help us better understand how microbes interact with their hosts.

## Disclosure statement

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