Environmental Science and Ecotechnology 21 (2024) 100431

Contents lists available at ScienceDirect

Environmental Science and Ecotechnology

journal homepage: www.journals.elsevier.com/environmental-science-and-ecotechnology/

Original Research

Biocontrol agents modulate phyllosphere microbiota interactions against pathogen *Pseudomonas syringae*

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ARTICLE INFO

Article history:
Received 21 October 2023
Received in revised form
13 May 2024
Accepted 15 May 2024
Available online xxx

Keywords:
Biological control
Metagenome
Transcriptome
Community structure
Pathogen resistance

ABSTRACT

The pathogen *Pseudomonas syringae*, responsible for a variety of diseases, poses a considerable threat to global crop yields. Emerging biocontrol strategies employ antagonistic microorganisms, utilizing phyllosphere microecology and systemic resistance to combat this disease. However, the interactions between phyllosphere microbial dynamics and the activation of the plant defense system remain poorly understood. Here we show significant alterations in phyllosphere microbiota structure and plant gene expression following the application of biocontrol agents. We reveal enhanced collaboration and integration of *Sphingomonas* and *Methylobacterium* within the microbial co-occurrence network. Notably, *Sphingomonas* inhibits *P. syringae* by disrupting pathogen chemotaxis and virulence. Additionally, both *Sphingomonas* and *Methylobacterium* activate plant defenses by upregulating pathogenesis-related gene expression through abscisic acid, ethylene, jasmonate acid, and salicylic acid signaling pathways. Our results highlighted that biocontrol agents promote plant health, from reconstructing beneficial microbial consortia to enhancing plant immunity. The findings enrich our comprehension of the synergistic interplays between phyllosphere microbiota and plant immunity, offering potential enhancements in biocontrol efficacy for crop protection.

1. Introduction

Pseudomonas syringae ranks among the top ten phytopathogens, causing various leaf diseases by entering wounds or natural openings such as stomata [1]. The pathovars of *P. syringae* have emerged as a global threat to agricultural production due to the wide range of plant hosts covering almost all economically important crop species. Multiple strategies have been developed to control the diseases and prevent crops from contracting *P. syringae*. For example, breeding disease-resistant varieties is a potential strategy. However, most are developed through the selection of wild plants or intraspecific hybridization based on existing resistant cultivars, leading to aimlessness and a narrow genetic background [2]. The current field *P. syringae* control relies heavily on copper formulations (e.g., copper sulfate) and antibiotics (e.g., streptomycin), while the long-term use of these agents presents issues with phytotoxicity, resistance in the pathogen, and changes in bacterial community structure [3]. Emna et al. isolated 43 *P. syringae* strains from Tunisian orchards and found that 67% were resistant to copper sulfate, 23% harbored the copper resistance genes *copABCDR* [4]. Furthermore, all approved antibiotics have faced resistance in some of their target pathogens [5]. The well-known consequence of resistance accumulation is a reduced

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https://doi.org/10.1016/j.ese.2024.100431

ability to prevent and treat bacterial infections. Moreover, copper and antibiotics exert strong selection pressure on the microbiota, which can alter the relative abundance of microbial species and interfere with interactions between different species and, ultimately, ecological functional stability. Massimo et al. highlighted that copper significantly changed the beta diversity of soil microbiota [6]. Numerous studies have shown that antibiotics lead to a reduction in bacterial diversity [7]. The rising awareness of copper formulations and antibiotic risks has increased the demand for safer alternatives. Biocontrol has been proposed as an environmentally friendly alternative for *P. syringae* suppression.

Biocontrol refers to exogenously added beneficial organisms and/or their products to limit the propagation of plant pathogens [8]. It generally increases the diversity of microbiota and the relative abundance of beneficial bacteria [9,10]. The meta-analysis has demonstrated that the biocontrol efficacy was significant and robust, and most importantly, the negative impacts on non-target species were much smaller than those on target species [11]. Currently, *Bacillus* spp., *Pseudomonas* spp., and *Pantoea* spp. are used as biocontrol agents for *P. syringae*, which can influence the virulence and/or epiphytic fitness of pathogens. Studies have shown that *Bacillus subtilis* forms biofilm and secretes surfactin [12], *Pseudomonas fluorescens* G20-18 produces cytokinin [13], and *P. syringae* pv. Syringae 22d/93 produces a siderophore that enhances the competitiveness of the antagonist [14]. In addition, 11% of all strains recovered from plants involved *Pseudomonas*, *Pantoea*, and *Erwinia* were found to interfere quorum sensing in *P. syringae* by limiting the iron availability [15]. By contrast, the researchers have found that various root-associated mutualists, including *Pseudomonas* and *Bacillus*, enhance plant defense against a broad range of pathogens by induced systemic resistance [16]. Plants can also recruit beneficial microbes to benefit generations by modifying exudation patterns in response to pathogens' attacks [17,18]. Together, these results indicate that changes in epiphytic microbiota, especially the enrichment of beneficial bacteria, may play a key role in inhibiting pathogen *P. syringae* and the induction of systemic resistance in plants. Although binary interactions between plants and individual strains of *P. syringae* and biocontrol agents have been extensively reported, how multi-species biocontrol agents affect the interactions of *P. syringae* with phyllosphere microbiota and how disturbed microbial communities impact plant metabolism are poorly understood. Multi-species biocontrol agents often demonstrate better control effectiveness than single-species biocontrol agents. Moreover, kasugamycin, an aminoglycoside antibiotic that acts only on plant pathogens, is the best alternative to streptomycin and gentamicin for *P. syringae* suppression [19]. Increasing the applications of kasugamycin reduced the total number and proportion of streptomycin-resistant bacteria in phyllosphere microbiota. Studies have shown that kasugamycin inhibited the epiphytic fitness of *P. syringae* [20]. Metabolomic profiling demonstrated that most antibiotic treatments negatively affect plant amino acid metabolism and the tricarboxylic acid (TCA) cycle [21]. However, research on the effects of kasugamycin on phyllosphere microbiota and plant metabolic function is limited.

We conducted a field plot experiment to better understand the potential mechanisms underlying disease control. We investigated the responses of phyllosphere microbiota and plants to the biocontrol agent and kasugamycin using metagenome and transcriptome analysis, respectively. We aimed to explore the influences of biocontrol agent and kasugamycin on (1) the structure and assembly of the phyllosphere microbial community, (2) the interaction between *P. syringae* and beneficial bacteria, and (3) the expression intensity of plant pathogenesis-related genes. Our results highlighted that the biocontrol agent could suppress *P. syringae* through synergic effects by increasing the competitiveness of the antagonist and stimulating the plant's immune system.

2. Materials and methods

2.1. Experimental design and sample collection

In this study, a field plot experiment was set up in Jiahe Country, Chenzhou, Hunan Province, China (25.676986° N 112.295213° E), and planted with Yunyan 87, a variety susceptible to *P. syringae* and known for causing wildfire disease. Upon observing initial disease spots, three different treatments were conducted: (i) CK, 10 L water without an agent; (ii) Km, 10 g kasugamycin (4% w/w, diluted 1000 times, Hanwei Bio-Technical & Science Co.,Ltd.), and (iii) BA, 1 g biocontrol agent BCA_B (diluted 10000 times), sprayed evenly on both sides of the tobacco leaf. The biocontrol agent, antagonistic to *P. syringae*, was screened from healthy leaves in our laboratory and was dominated by *Bacillus* (87.74%), *Alcaligenes* (7.69%), *Pseudochrobactrum* (2.86%), and *Achromobacter* (1.05 %) [10]. Each treatment contains 90 plants (three columns, 1.2 m apart; 30 rows, 0.5 m apart) and performed three replicates. A total of nine experimental plots were randomly distributed. Five plants were labeled randomly in each plot, and the infection rate and disease index of wildfire disease were investigated after seven days. The infection rate and disease index are calculated according to the following equations (Grade and investigation method of tobacco diseases and insect pests GB/T 23222-2008):

$$\text{Infection rate} = \frac{ni}{nt} \times 100\% \quad (1)$$

where ni is the number of infected plant leaves and nt is the total number of investigated plant leaves.

$$\text{Disease index} = \frac{\sum r \times ni}{nt \times R} \times 100 \quad (2)$$

where r is the degree of disease infection, ni is the number of infected plant leaves corresponding to the grade of r, nt is the total number of investigated plant leaves, and R is the highest degree of disease infection. The degrees of disease infection (r) were assigned to six grades (0, 1, 3, 5, 7, and 9).

Three leaves from the five labeled plants were randomly picked in each plot, and microbial samples were collected by washing each leaf surface with phosphate-buffered saline (PBS) buffer. The leaves were then frozen using liquid nitrogen and transported in a dry-ice pack to the laboratory. The microbial and plan samples were stored at −80 °C until DNA and RNA extraction.

2.2. DNA extraction, library construction, and sequencing

Phyllosphere microbial DNA was extracted using E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer's instructions. The library construction and sequencing were conducted at the LC-Bio Technology Co.,Ltd., Hangzhou, China. Genomic libraries were prepared with the TruSeq Nano DNA Library Preparation Kit (#FC-121-4001, Illumina, USA) following the manufacturer's instructions. Sequencing was performed on Illumina Novaseq 6000.

2.3. RNA isolation, library construction, and sequencing

Plant RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The concentration and purity of RNA were quantified using NanoDrop ND-

1000 (NanoDrop, Wilmington, DE, USA). The poly(A) RNA was specifically captured by two rounds of purification using Dynabeads Oligo (dT)25-61005 (Thermo Fisher, CA, USA). Then, the poly(A) RNA was fragmented into small pieces using a magnesium RNA fragmentation module (NEB, cat. e6150, USA) under 94 °C for 5−7min. The cleaved RNA fragments were reverse-transcribed to create the cDNA library with a final size of 300 ± 50 bp by SuperScrip II Reverse Transcriptase (Invitrogen, cat. 1896649, USA). Finally, PE150 sequencing was performed on an Illumina Novaseq 6000 (LC-Bio Technology Co.,Ltd., Hangzhou, China) following the vendor's recommended protocol.

2.4. Sequencing reads assembly, taxonomic, and functional assignment

Metagenome raw sequencing reads were cleaned to exclude adapter sequences, low-quality sequences (fastq [22]), and contaminated DNA, including host (bowtie2 [23]). The clean reads from all samples were pooled together and assembled by Megahit (v1.1.3) [24], and then gene prediction was performed by Prodigal (v2.6.3) with parameter "-p meta" [25]. A non-redundant gene catalog was constructed using the predicted gene models by cd-hit-est (v4.8.1) [26]. Unigene abundance for a certain sample was estimated by TPM based on the number of aligned reads by bowtie2 (v2.2.0).

Transcriptome raw sequencing reads were cleaned to exclude adapter and low-quality sequences using fastq [22]. Then, the clean reads were assembled by Trinity (v2.8.5) [27], and transcripts were clustered using cd-hit-est. The transcript expression level was measured by TPM calculated by salmon (v0.14.1) [28].

Taxonomic assignments were made by mOTUs (v3.0.2) [29]. The functional annotation of unigenes was made based on DIAMOND alignment against eggNOG (v5) [30,31]. Genus, species, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and KEGG Orthology (KO) relative abundances were calculated by summing the abundance of the respective genes belonging to each category per sample.

2.5. Statistical analysis

Choa1, Shannon, and Simpson index was used for diversity analysis. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity values was used to evaluate the overall differences in microbial communities and plant expression structures. The significant taxonomic and functional differences between groups were determined by the analysis of variance (ANOVA) tests and adjusted by the false discovery rate (FDR) methods for multiple tests. Co-occurrence networks were constructed according to Spearman's rank correlation coefficient between genera, and the networks were visualized with Gephi 0.9.2. Infer community assembly mechanisms by phylogenetic-bin-based null model analysis (iCAMP) was used to quantify the microbial community assembly [32].

3. Results and discussion

3.1. Plant health

Infection rate and disease index were used to describe the degree of plant disease infection. The infection rate and disease index in treatment groups Km and BA showed similar trends, which decreased compared with the control group CK (Fig. S1). However, BA's average infection rate and disease index were lower than in Km. The results suggest that kasugamycin, especially biocontrol agents, plays a positive role in inhibiting plant disease.

3.2. Phyllosphere microbial diversity and interactions

A total of 394 OTUs were identified in the phyllosphere microbial community across three groups. The microbial richness (Chao1 index) of both the Km and BA groups was significantly lower than that of the CK group ($P < 0.05$; Table S1). The results of ADONIS showed that there was a significant difference in the structure of the microbial community. Furthermore, the principal coordinate analysis (PCoA) confirmed that 53.1% of the variance in the microbial community could be explained by two PCoA axes. Specifically, microbial communities of CK and BA groups were distinctly separated by the PCoA1 axis ($P < 0.05$) but not by the PCo2 axis ($P > 0.05$), while that of CK and Km could not be separated by these two axes ($P > 0.05$; Fig. 1a). Moreover, there were significant differences in the composition of the phyllosphere microbial community at the genus level. For example, *Pseudomonas*, *Sphingomonas*, and *Methylobacterium* were dominant taxa, jointly accounting for over 60% of relative abundance in the microbial community, but they each showed a remarkable variation across the three groups. Specifically, the relative abundance of *Sphingomonas* and *Methylobacterium* in the BA group was significantly higher than that in the other two groups, while *Pseudomonas* was the opposite (Fig. 1b). Studies have suggested that the members of *Sphingomonas* exhibit a striking plant-protective effect by diminishing pathogen growth through direct competition with pathogen *Pseudomonas syringae* for carbon sources [33]. Meanwhile, *Methylobacterium* contributes to the defense system of plants against the pathogen *P. syringae* [34].

Beyond microbial community structure and composition differences, biodiversity also encompasses the complex microbial interactions among species [35]. A co-occurrence network of microbial species was constructed. Then, the subnetworks belonging to three groups were extracted to analyze the influences of the biocontrol agent on phyllosphere microbial interactions ($r > 0.55$, $P < 0.05$). *Pseudomonas*, *Sphingomonas*, and *Methylobacterium* were dominant in three subnetworks. The relative proportions of *Pseudomonas* in BA (5.8%) and Km (11.7%) subnetwork nodes were lower than those in CK groups (19.8%) (Fig. 1c). Conversely, the relative proportions of *Sphingomonas* and *Methylobacterium* increased in the subnetworks Km (33.0% and 34.9%) and BA (21.3% and 17.4%) compared with CK (13.4% and 8.0%). In addition, more than 90% of the links in all the subnetworks were positive, implying greater cooperation among community members [36]. The effects of the biocontrol agent on network topological properties were also investigated (Table S2). We observed that the average path length between all paired nodes in the BA subnetwork was significantly shorter than that of the CK subnetwork, which suggests that the BA network has a higher efficiency [37]. We also found that the average degree, which indicates a complex relationship among network members, was significantly lower in the Km subnetwork than in the CK subnetwork [38]. These results suggested a difference in the performance of antibiotics and microbial agents in disease prevention by altering microbial interactions. Kasugamycin reduced the biodiversity of phyllosphere microorganisms and the abundance of pathogenic bacteria, while biocontrol agents specifically enriched antagonistic bacteria and increased cooperation among beneficial bacteria to inhibit the growth of pathogenic bacteria.

3.3. Phyllosphere microbial community assembly

To explore the roles played by the biocontrol agent in microbial community assembly, we first divided 394 OTUs into nine phylogenetic bins (Fig. 2a). We calculated the relative importance (RI) of ecological processes. Overall, there were merely two processes,

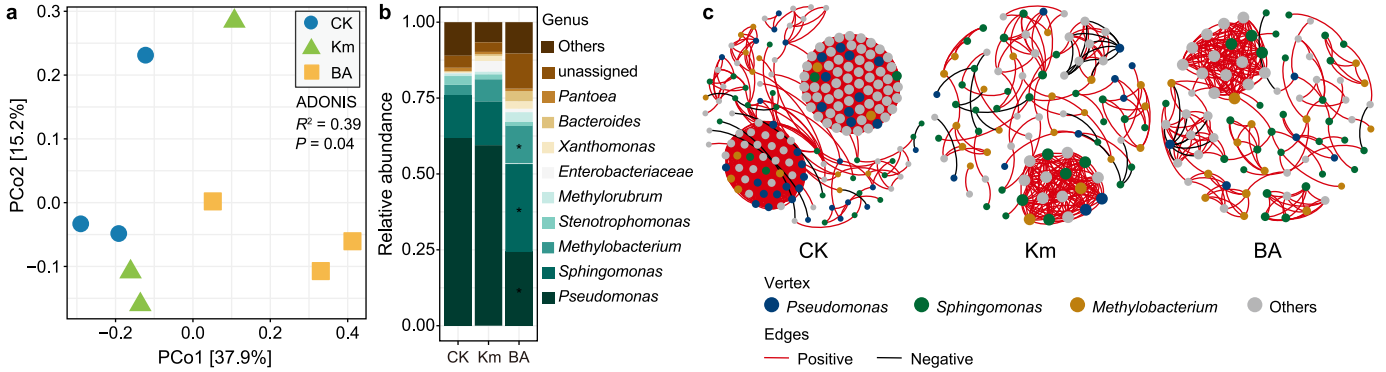

Fig. 1. Comparison of phyllosphere microbiome in different groups. **a**, Composition at genus rank. **b**, The principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarities at the OTU level. **c**, Microbial interactions.

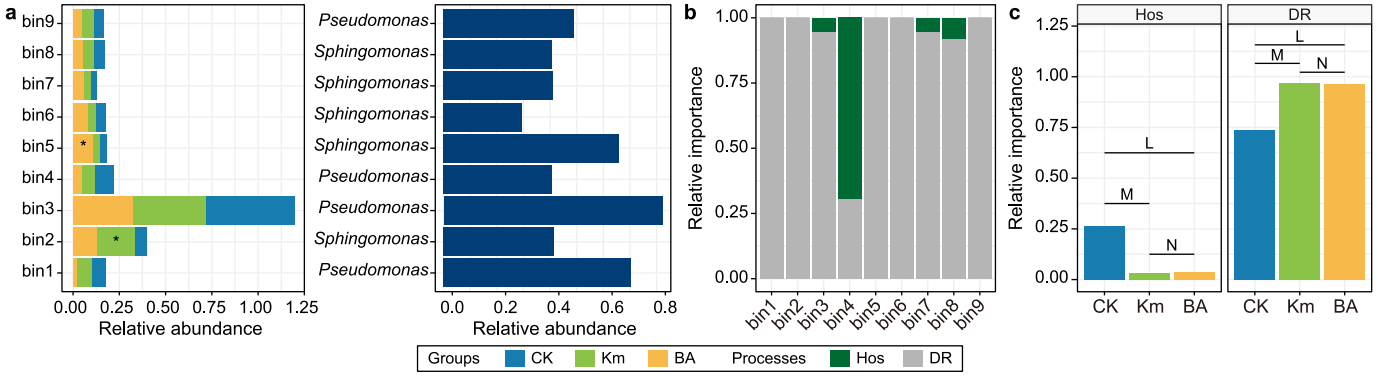

Fig. 2. Phyllosphere microbial community assembly mechanism. **a**, Relative abundance of each bin (left) and the genus with the greatest relative abundance in each bin (right). **b**, Relative importance of homogeneous selection and drift in the assembly of each bin. **c**, Relative importance of different ecological processes based on iCAMP.

including homogeneous selection (HoS) and drift (DR), shaping the assembly of phylogenetic bins. The assembly of phylogenetic bin4 was primarily driven by homogeneous selection (RI = 69.4%), while that of the rest of the eight phylogenetic bins was dominated by drift (RI = 73.7–97.0%) (Fig. 2b). This indicates that the members of phylogenetic bin4 are more susceptible to environmental or biological selection than members of other phylogenetic bins. Phylogenetic bin4 contains major members of *Pseudomonas*, accounting for 40.7% of the relative proportion, and their relative abundances in BA and Km groups were lower than that in the CK group (Fig. 2a). These results imply that biocontrol agents may primarily target pathogen species in phylogenetic bin4 for disease control. Additionally, as for phylogenetic bin2 and bin5, which are mainly composed of *Sphingomonas* members, their relative abundances in the BA and Km groups were significantly higher than in the CK group ($P < 0.05$). After calculating the weighted accumulation of ecological processes of nine phylogenetic bins for the microbial community of each sample, we found that the relative importance of homogeneous selection in community assembly in BA and Km groups was lower than CK groups with large (Cohen's $|d| = 0.95$) and medium (0.77) effect size, respectively (Fig. 2c). It suggests that the use of biocontrol agent or kasugamycin may inhibit those sensitive species, which weakens the constraints of environmental or biological selection in community assembly.

3.4. Metabolic potential of phyllosphere microbial community

A total of 2111781 genes were identified from microbial metagenomes and clustered into 912736 gene families (clustering threshold 0.95, word size 10), and 94192 gene families were successfully assigned with KEGG orthology. Our results showed that the within-group distance of gene families in the CK group was significantly larger than that in BA and Km groups (Fig. 3a), which implies that there was a convergent response in function structures of the microbial community to biocontrol agent or kasugamycin treatments. The virulence factor protein genes were downloaded from the *Pseudomonas syringae* Genome Resources.[1] The orthologs were searched, and 11 virulence factors were obtained for *P. syringae* (Fig. S2). Among them, the relative abundance of *hopI1*, *avrE1*, *hopQ1*, *hopAG1*, *hopB1*, *hopQ1-1*, *avrD1*, and *hopAV1* were higher in CK group, while *hopAG::ISPssy*, *hopD1*, and *hopBH1* were higher in Km group. According to the KEGG functional profiles, we found that the plant-pathogen interactions of the KEGG pathway showed a significant variance across three groups ($P < 0.05$) (Fig. 3b). In this KEGG pathway, the relative abundance of genes encoding elongation factor Tu (elf18, K02358) and heat shock protein 90 (HSP90, K04079) in BA group was significantly lower than that in CK group. elf18 is a typical pathogen-associated molecular pattern (PAMP) in which plants switch on stomatal defense to reduce bacterial entry through stomata by detecting PAMPs [39]. To defeat stomatal defense, *P. syringae* uses type III secretion system (T3SS) effector proteins to overcome PAMP-induced stomatal closure [40]. HSP90 is essential for the virulence of T3SS effector HopI1. Plant stomatal defense is disrupted by HopI1, which directly binds HSP90 to form large complexes that transport pathogens to

[1] http://pseudomonas-syringae.org/.

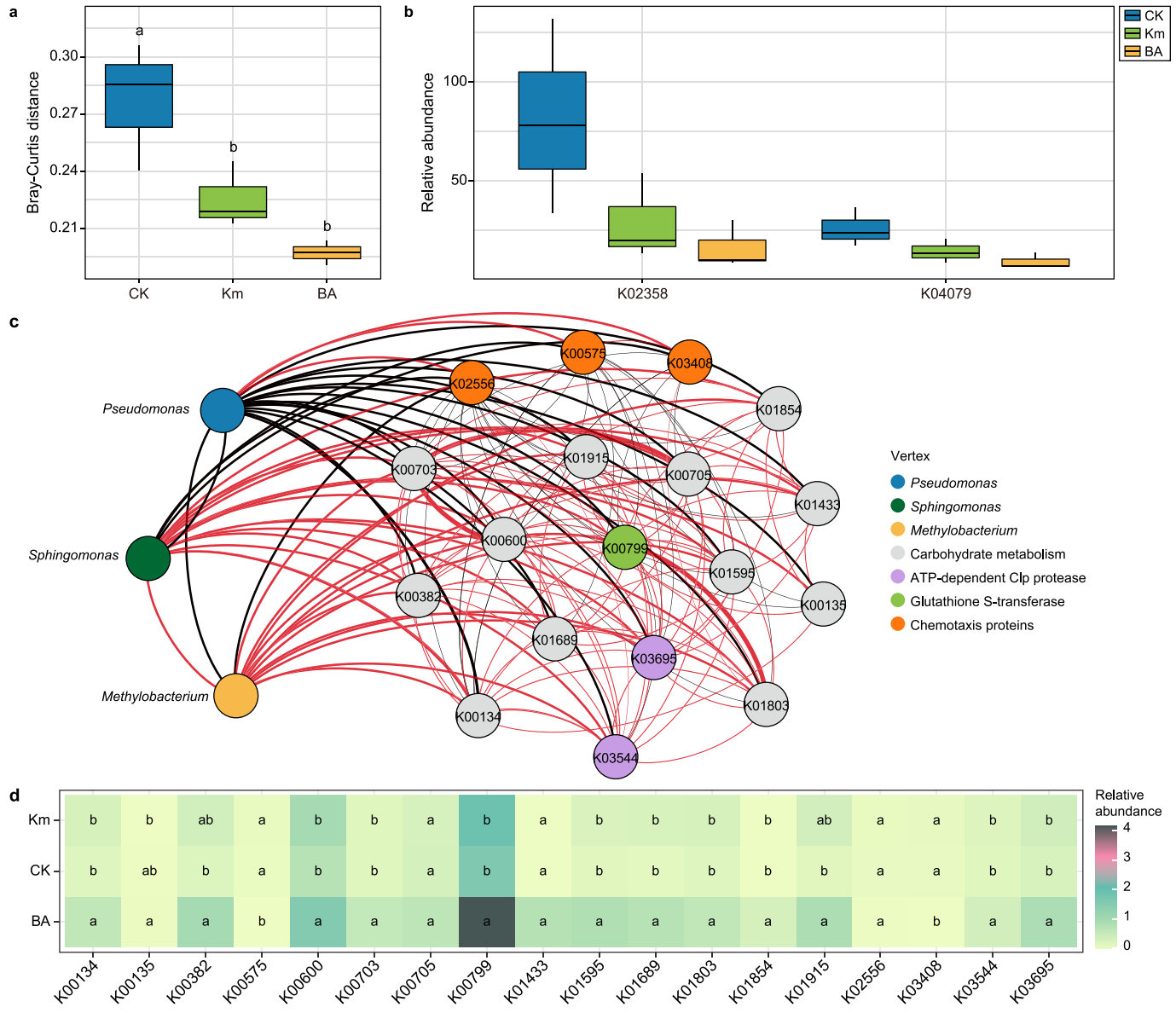

Fig. 3. Effect of biocontrol agents on phyllosphere microbial functional genes. **a**, Bray-Curtis distance in different groups. **b**, Relative abundance of genes involved in plant-pathogen interaction. **c**, Correlation network based on the relative abundance of three key taxa and 18 functional genes. **d**, Heatmap showing the relative abundance of the 18 genes.

chloroplasts [41].

To further explore how the key genera, including *Pseudomonas*, *Sphingomonas*, and *Methylobacterium* identified in sections 3.1 and 3.2 modulate function structure in the phyllosphere microbial community, we constructed a correlation network based on the relative abundance of the above three genera and KEGG orthologies ($P < 0.01$, $r \geq 0.7$) (Fig. 3c). We found that three genera were primarily connected with 18 orthologies, which were affiliated to carbohydrate metabolic enzyme, adenosine triphosphate (ATP)-dependent Clp protease, glutathione S-transferase and chemotaxis proteins. For instance, *Sphingomonas* was positively correlated with 12 genes encoding carbohydrate metabolic enzyme, all of which had the highest relative abundance in BA group (Fig. 3d), including butanoate metabolism (K00135), galactose metabolism (K01854), glycolysis/gluconeogenesis (K00382, K00134, K01803, K01689), glyoxylate and dicarboxylate metabolism (K00600, K01915, K01433), pyruvate metabolism (K01595), and starch and sucrose metabolism (K00705, K00703). This suggested that *Sphingomonas* spp. may inhibit the growth of *P. syringae* in the phyllosphere through the competition for carbon sources [33]. Consistently, glutathione S-transferase (K00799) and two ATP-binding subunits of ATP-dependent Clp protease (K03695 and K03544) positively associated with *Sphingomonas*, also have the highest relative abundance in the BA group. ATP-dependent Clp protease plays an indispensable role in motility, biofilm formation, and stress adaptation [42], and glutathione S-transferase is recognized as the detoxification enzymes involved in many pathologic and physiological processes [43], both of which may further improve the fitness and competitiveness of *Sphingomonas* during inhibiting pathogen *P. syringae*. Meanwhile, chemotaxis proteins MotA (K02556), CheR (K00575), and CheW (K03408), which were positively related to *Pseudomonas*, showed the lowest relative abundance in the BA group. These chemotaxis proteins contributed to virulence and pathogenic fitness in *P. syringae* [44,45]. This implies that the biocontrol agent may interfere with the chemotaxis system of *P. syringae* for disease control.

3.5. Response of plant transcriptome

A total of 2387823 genes were identified from plant transcriptome and clustered into 754199 gene families, and 590813 gene families were successfully assigned with KEGG orthology. Our results showed that gene diversity in the Km groups was significantly lower than that in the CK group ($P < 0.05$) (Table S3), but there was no significant difference in gene diversity between the BA and CK groups. However, PCoA demonstrated that CK and BA groups were separated by PCoA1 and PCoA2 axis, which explained 67.1% of the variance in plant expression (Fig. S3; $P < 0.05$). This indicates that the biocontrol agent regulates the functional pattern instead of gene diversity in plant expression. According to KEGG functional profiles, we found that the expression intensity of plant genes involved in hormone signal transduction was significantly increased 1.7 times in the BA group (1102) compared with that in the CK group (403) (Fig. 4a; $P < 0.05$). This increase primarily involved four hormones, including abscisic acid (ABA), ethylene (ET), jasmonate acid (JA), and salicylic acid (SA). Specifically, ET and SA signal transduction pathways play a fundamental role in disease resistance and building immunity when faced with invasive pathogens [46,47]. Compared with the CK group, the expression of gene encoding ethylene-insensitive protein 3 (EIN3, K14514) in BA group (13.4) was up-regulated by 1.7 times within ET response pathway, while that of regulatory protein NPR1 (NPR1, K14508) for SA in BA group (39.9) was down-regulated 15.4% (Fig. 4b). As for JA signaling pathway of plant host, it was exploited by pathogen *P. syringae* to promote its virulence [48], and the expression of genes encoding jasmonate ZIM domain-containing protein (JAZ, K13464) in BA group (549.1) was up-regulated by 9.8 times than that in CK group.

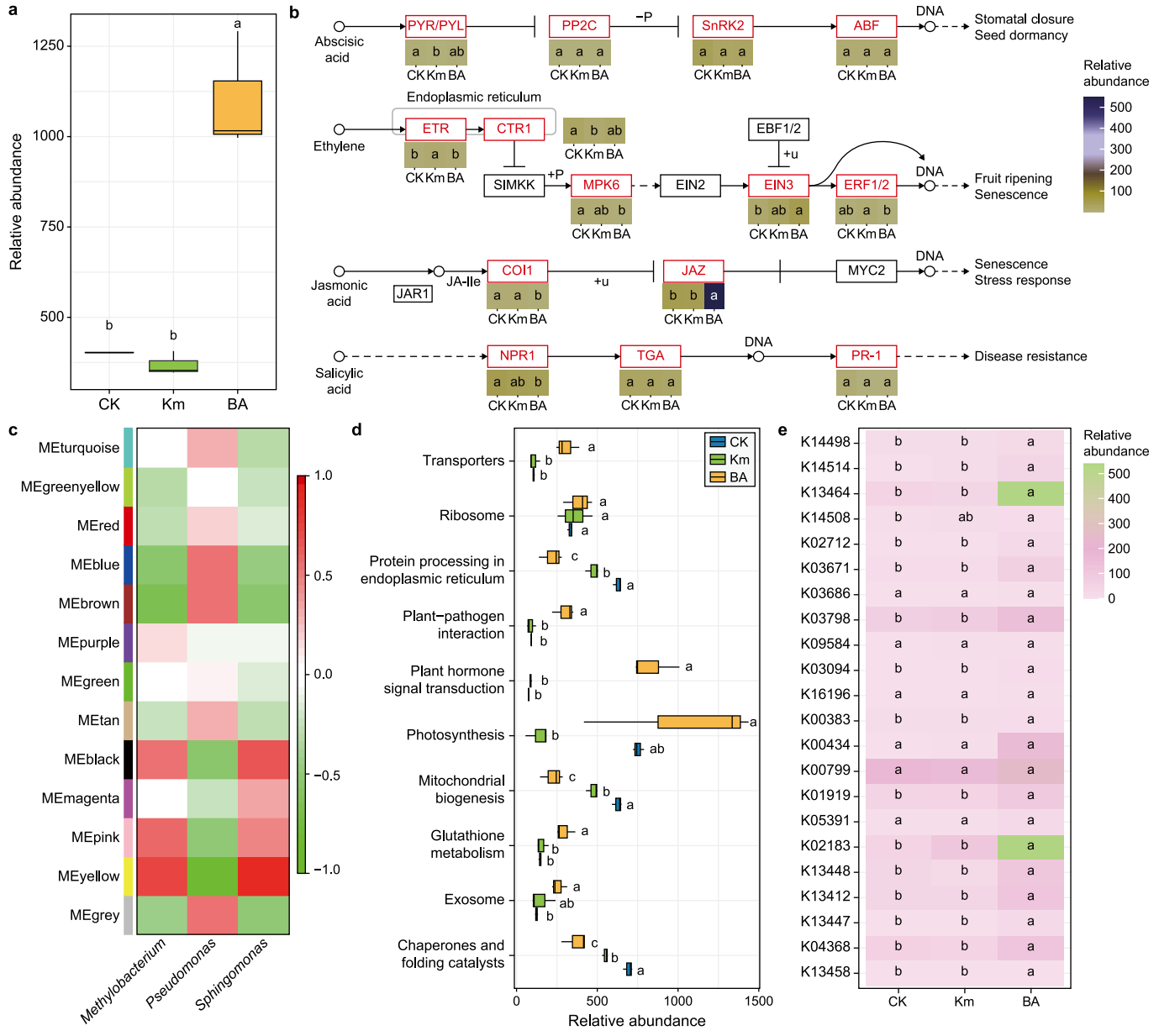

Fig. 4. Effect of biocontrol agents on plant gene expression. **a**, Relative abundance of genes involved in plant hormone signal transduction. **b**, Relative abundance of genes for abscisic acid (ABA), ethylene (ET), jasmonate acid (JA), and salicylic acid (SA) signaling pathways. **c**, Correlation analysis of 12 modules detected in gene co-expression network and three key genera. **d**, Relative abundance of KEGG metabolic pathway of the yellow module. **e**, Heatmap showing the relative abundance of the KO.

These results suggested that biocontrol agent influences plant hormone-responsive pathways during pathogen suppression.

Furthermore, we constructed a weighted gene co-expression network based on the expression intensity of plant functional genes. A total of 12 network modules were detected and were related to three key genera, including *Pseudomonas*, *Sphingomonas*, and *Methylobacterium*. Notably, one module, MEyellow, was significantly correlated with the above three key genera (Fig. 4c; $P < 0.05$), which consisted of 352 genes involved in 130 KEGG pathways at level 3 (Fig. S4). Among them, the relative proportion of genes from the top ten KEGG pathways, including photosynthesis, plant hormone signal transduction, chaperones and folding catalysts, protein processing in the endoplasmic reticulum, mitochondrial biogenesis, transporters, plant-pathogen interaction, glutathione metabolism, exosome and ribosome (Fig. 4d), accounted for over 54.6% of the node in MEyellow module. Plant hormones such as ABA, ET, JA, and SA regulate plant defense responses against pathogens [49]. It has been demonstrated that injection of SA into plants increased photosynthetic rate [50], glutathione content [51], and Ca^{+} signaling treansduction [52]. Moreover, the SA receptor NPR1 directly controls the expression of the protein secretory pathway genes [53]. These results indicate that plant pathogen resistance mediated by hormone-responsive genes is closely interconnected with photosynthesis, chaperones, folding catalysts, protein processing in the endoplasmic reticulum, glutathione metabolism, and plant-pathogen interaction.

Specifically, compared with CK group, the genes encoding serine/threonine-protein kinase SRK2 (SNRK2, K14498), EIN3 (K14514), JAZ (K13464), and NPR1 (K14508) involved in plant hormone signal transduction were all significantly up-regulated in BA group (Fig. 4e). SNRK2 kinases of ABA signaling inhibit the initiation and proliferation of the stomatal precursors in plants [54]. EIN3 proteins involve ET-mediated induction of defense-related effector genes, and cross-talk occurs with other defense response genes regulated by JA and SA [55], which are JAZ and NPR1 in our results. JAZ proteins of JA signaling function to repress the expression of JA-responsive genes [56,57]. Similarly, NPR1 proteins are required for SA-induced plant resistance against pathogens [58]. Pathogen *P. syringae* virulence factors have been reported to impair plant stomatal defense and SA-mediated immune response through JA-SA antagonism [1]. These results suggest that the biocontrol agent enhances plant resistance against *P. syringae* by inhibiting JA signaling and activating ABA, ET, and SA-mediated stomatal defense and pathogenesis-related gene expression. In addition, the genes encoding photosystem II PsbK protein (K02712) for photosynthesis, the genes encoding thioredoxin (K03671), molecular chaperone DnaJ (K03686), cell division protease FtsH (K03798), and protein disulfide-isomerase A6 (K09584) for chaperones and folding catalysts, the genes encoding S-phase kinase-associated protein 1 (K03094) and eukaryotic translation initiation factor 2-alpha kinase 4 (K16196) for protein processing in the endoplasmic reticulum, the genes encoding glutathione reductase (K00383), L-ascorbate peroxidase (K00434), glutathione S-transferase (K00799), and glutamate-cysteine ligase (K01919) for glutathione metabolism, and the genes encoding cyclic nucleotide gated channel (K05391), calmodulin (K02183, K13448), calcium-dependent protein kinase (K13412), respiratory burst oxidase (K13447), mitogen-activated protein kinase kinase 1 (K04368), and disease resistance protein (K13458) for plant-pathogen interaction all significantly up-regulated in BA group and are essential for plant systemic acquired resistance ($P < 0.05$).

3.6. Mechanisms underlying biocontrol agent against plant disease

Biocontrol agents are recognized as an environmentally safe method to prevent plant diseases because their negative impacts on non-target species were much smaller than on target species [11]. Many studies have approved that biocontrol agents do not significantly affect plant microbial community diversity or composition [59,60]. The results regarding microbial community structure in this study showed that the relative abundance of *Bacillus* accounted for less than 1% in all three treatment groups, and there was no significant difference between the groups. This indicates that the functional bacteria in the biocontrol agent do not colonize the leaf surface. However, there were significant changes ($P < 0.05$) in the relative abundance of dominant taxa *Pseudomonas*, *Sphingomonas*, and *Methylobacterium*. We suggested that biocontrol agents combat disease by influencing bacterial interactions and replacing ecological niches to form new functional modules [61]. The increase in the abundance of beneficial *Sphingomonas* and *Methylobacterium* replaces the niche of *Pseudomonas*, aiding in preventing leaf diseases. These beneficial bacteria can prevent plant diseases through synergistic effects of nutritional competition and plant resistance induction [62]. For example, *Micromonospora* obtained from the root nodules of legumes has been observed to have direct inhibitory effects on several pathogenic fungi. In addition, this microbe also induces tomato JA signaling to defend against the fungal pathogen *Botrytis cinerea* [63]. Our results showed that biocontrol agents increased the fitness and competitiveness associated with *Sphingomonas* while decreasing the virulence and fitness associated with *Pseudomonas*. From this, we infer that a biocontrol agent inhibits the growth of *P. syringae* by enhancing competitiveness for beneficial *Sphingomonas* (see Fig. 5).

In addition, beneficial bacteria induce the host immune response through microbe-associated molecular patterns. Systemic resistance induced by different beneficials, such as *Pseudomonas* spp. and *Bacillus* spp., is regulated by similar JA-dependent and ET-dependent signaling [34,64]. This study suggested that *Sphingomonas* and *Methylobacterium* can induce plant resistance response through four plant hormones, including JA, SA, ET, and ABA. By inhibiting JA signaling and stimulating ABA, ET, and SA signaling, it activates stomatal defense and pathogenesis-related gene expression. Moreover, plant pathogen resistance genes mediated by the above four hormones were found to be co-expressed with genes related to photosynthesis and protein secretory pathways. Photosynthesis provides a sustained energy supply for plant defense, while protein secretory promotes the secretion of pathogenesis-related genes [53,65].

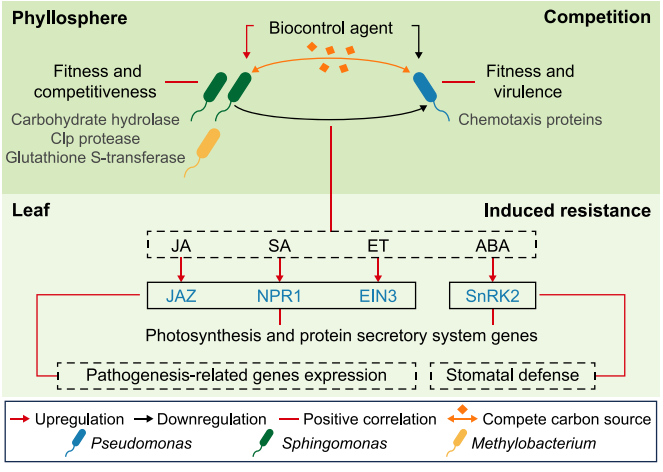

Fig. 5. Mechanisms of biocontrol agent of phytopathogens by nutritional competition and plant resistance induction.

4. Conclusions

In conclusion, this study provides an in-depth understanding of the substantial impact of biocontrol agent treatment on phyllosphere microbiota and plants in the field environment. Biocontrol agents prevent leaf diseases by increasing the abundance of beneficial bacteria and replacing pathogenic bacteria niches. Through comprehensive multi-omics analysis, we reveal the mechanism of synergistic action of beneficial bacteria and plants against pathogens. Biocontrol agents inhibited the growth of *Pseudomonas syringae* by increasing carbohydrate hydrolase, adenosine triphosphate (ATP)-dependent Clp protease, and glutathione S-transferase of beneficial bacteria and intervening chemotaxis system of *P. syringae*. Meanwhile, biocontrol agents induced plant stomatal defense and pathogenesis-related gene expression by inhibiting jasmonate acid (JA) signaling and stimulating abscisic acid (ABA), ethylene (ET), and salicylic acid (SA) signaling. Ultimately, the insights from this study will inform the development of guidelines for strategic biocontrol agent application.

CRediT authorship contribution statement

Zhaoyue Yang: Conceptualization, Visualization, Writing - Original Draft. **Tianbo Liu:** Conceptualization, Validation. **Jianqiang Fan:** Investigation. **Yiqiang Chen:** Investigation. **Shaolong Wu:** Resources. **Jingjing Li:** Resources. **Zhenghua Liu:** Writing - Review & Editing. **Zhendong Yang:** Writing - Review & Editing. **Liangzhi Li:** Methodology. **Suoni Liu:** Methodology. **Hongwu Yang:** Investigation. **Huaqun Yin:** Conceptualization, Project Administration. **Delong Meng:** Project Administration, Writing - Review & Editing. **Qianjun Tang:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financially supported by the Key Project of Science and Technology Project of China National Tobacco Corporation (110202101027) [LS-11], the Key Research Project of Hunan Province (2023NK2019), the Natural Science Foundation of Changsha (kq2202089), and the International Scientific and Technological Cooperation Base of Hunan (2018WK4019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2024.100431.

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