

Host genotype shapes microbiome reassembly and recruitment of a maculosin-associated biocontrol bacterium in alfalfa leaf blight caused by *Fusarium citri*

Muzhapaer Tuluhong

Northeast Agricultural University

Baiji Wang

Northeast Agricultural University

Qianhan Zhao

Northeast Agricultural University

Meiqi Mu

Sun Yat-sen University

Xinyi Zhang

Northeast Agricultural University

Xiangpeng Kong

Northeast Agricultural University

Yuying Xiao

Northeast Agricultural University

Rui Qiu

Northeast Agricultural University

Wenjie Zhao

Northeast Agricultural University

Guowen Cui

cgw603@163.com

Northeast Agricultural University

Article

Keywords: alfalfa leaf blight, *Fusarium citri*, phyllosphere microbiome, endophytic microbiota, *Pantoea agglomerans*, maculosin

Posted Date: June 9th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9329404/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Alfalfa leaf blight threatens forage production, yet the links among pathogen identity, host resistance, and microbiome-mediated suppression remain poorly resolved. Here, we identified *Fusarium citri* as the causal agent of alfalfa leaf blight in Heilongjiang Province, China, based on consistent isolation from symptomatic tissues, pathogenicity assays, re-isolation, and multilocus phylogenetic analysis. Screening of 33 alfalfa cultivars revealed marked variation in resistance and identified Zhongmu 3 and Juneng 7 as contrasting resistant and susceptible genotypes, respectively. Compared with the susceptible cultivar, the resistant cultivar developed milder symptoms, retained greater tissue integrity, and displayed a distinct defense profile characterized by strong PAL induction. *F. citri* infection also drove pronounced reassembly of both epiphytic and endophytic bacterial communities in a host genotype-dependent manner, including shifts in community composition, co-occurrence network structure, and predicted functional potential, with enrichment of *Pantoea* and *Pseudomonas* in the resistant background. Guided by these microbiome changes, we isolated *Pantoea agglomerans* MX28, which showed strong antagonistic activity against *F. citri*. Metabolomic, genomic, and pot experiments further linked MX28-mediated disease suppression to maculosin-associated antifungal activity, putative nutrient competition, and activation of host defense responses. Together, these findings support an integrated host–microbiome–pathogen model for alfalfa leaf blight and identify MX28 as a promising candidate for microbiome-assisted disease management.

Introduction

Alfalfa (*Medicago sativa* L.), a globally prominent perennial leguminous forage crop, plays a pivotal role in livestock husbandry due to its high nutritional value and broad palatability ¹. Beyond its importance as feed, alfalfa improves soil fertility through biological nitrogen fixation and enhances soil structure via its deep root system ^{2,3}. Its drought tolerance and capacity to mitigate soil erosion further highlight its ecological value. Globally, alfalfa is cultivated on approximately 40 million hectares, supporting livestock industries in more than 80 countries ⁴. However, climate change and increasingly intensive agricultural practices have been accompanied by more frequent and severe disease outbreaks in major alfalfa-producing regions ⁵. Among foliar diseases, alfalfa leaf blight has emerged as a growing threat because it causes rapid tissue necrosis, compromises photosynthetic capacity, and ultimately reduces forage yield and quality ^{1,6,7}. Despite its increasing importance, the etiology, infection biology, and host resistance basis of alfalfa leaf blight remain insufficiently characterized, hindering the development of effective and sustainable control strategies.

Fusarium species are globally distributed phytopathogenic fungi responsible for economically important diseases across diverse crops ^{8,9}. Accurate identification of *Fusarium* pathogens is often challenging because closely related taxa can exhibit highly similar colony and conidial morphologies, which may lead to misclassification when relying solely on traditional culture-based traits ^{10,11}. The adoption of molecular phylogenetic approaches has therefore become essential for robust diagnosis and

epidemiological inference. Multilocus sequence analysis using markers such as the internal transcribed spacer (ITS), translation elongation factor 1- α (*TEF1- α*), and the second largest subunit of RNA polymerase II (*RPB2*) has been widely applied to resolve species boundaries and improve taxonomic assignment within *Fusarium* ¹². Importantly, even when a single causal species is implicated, pathogen populations can still display substantial intraspecific variation in aggressiveness and infection dynamics, with consequences for disease development and host responses ¹³. Thus, linking alfalfa leaf blight to a single causal pathogen—*Fusarium citri*—requires integrative evidence from morphology, multilocus phylogenetics, and pathogenicity assays, together with a reproducible inoculation framework to support downstream resistance and microbiome analyses.

The growing threat of alfalfa leaf blight underscores the urgency of implementing effective and sustainable disease management strategies. Although chemical control remains widely used, its long-term efficacy can be unstable and it may pose risks of environmental contamination ¹⁴. Consequently, breeding and deploying disease-resistant alfalfa cultivars is increasingly viewed as a cornerstone of durable control ¹⁵. However, the mechanisms underlying cultivar-dependent resistance to *F. citri* infection remain poorly understood, which limits progress in resistance breeding and the rational design of complementary interventions. In particular, how resistant and susceptible cultivars differ in infection progression at the tissue level and in the timing and magnitude of defense activation has not been fully resolved ¹⁶⁻¹⁸. Addressing these knowledge gaps is critical for building a mechanistic foundation for improved alfalfa resistance and for integrating host-based strategies with additional layers of protection such as microbiome-associated disease suppression.

Plant health is not solely determined by host genetics and pathogen pressure but is also shaped by the resident microbiome that occupies aboveground tissues. The phyllosphere (epiphytic) and endosphere (endophytic) communities can restrict pathogen establishment through niche pre-emption, resource competition, antibiosis, and microbe-triggered immunity, thereby forming a disease-suppressive ecological state ¹⁹⁻²¹. Conversely, invasion by a foliar pathogen can perturb community assembly and interaction networks, leading to dysbiosis and increased opportunities for secondary colonizers ^{20,22}. Importantly, microbiome contributions to disease outcomes are often encoded not only in taxonomic shifts but also in network topology and functional potential, including pathways involved in secondary metabolism, signaling, and membrane transport ^{23,24}. In alfalfa leaf blight caused by *Fusarium citri*, resistance varies substantially among cultivars, yet it remains unclear whether resistant and susceptible hosts differentially recruit or maintain epiphytic and endophytic bacteria during *F. citri* infection, and whether specific keystone taxa and their metabolites contribute causally to disease suppression. Addressing these gaps requires integrating pathogen biology with microbiome profiling, ecological network analysis, and mechanistic validation of candidate antagonists and antifungal molecules.

Here, we identify *Fusarium citri* as the causal agent of alfalfa leaf blight and show that cultivar-dependent resistance is accompanied by marked reassembly of the leaf microbiome. By comparing resistant and susceptible cultivars, we resolve pathogen-induced shifts in epiphytic and endophytic bacterial

communities, their interaction networks, and predicted functional traits. We further identify a resistance-associated antagonistic bacterium and combine genomic, metabolomic, and functional evidence with host anatomical and defense analyses to uncover mechanisms of microbiome-linked disease suppression. These findings establish a mechanistic link between host resistance, microbiome reassembly, and suppression of alfalfa leaf blight.

Results

Etiological identification of alfalfa leaf blight and confirmation of *F. citri* as the causal agent

Alfalfa leaf blight symptoms in the field began as water-soaked or chlorotic areas, often from leaf margins and along veins, and rapidly progressed to irregular yellow-brown blighted patches. Lesions expanded and coalesced, leading to extensive necrosis, leaf desiccation, and premature senescence (Fig. 1a, f). To identify the causal agent, symptomatic leaves were collected from major alfalfa-producing areas in Heilongjiang Province (10 locations; n = 250) (Additional file 1: Fig. S1). Following surface sterilization and incubation of lesion-margin tissues on PDA, a total of 92 fungal isolates were obtained and purified, among which 61 were preliminarily identified as *F. citri*. A subset of representative isolates (MYK1-MYK5) was selected for morphological characterization and molecular identification. Pathogenicity assays verified that *F. citri* induced typical alfalfa leaf blight and satisfied Koch's postulates (Fig. 1g). At 7 dpi, symptoms aggravated, with a mean lesion diameter of 12.58 mm, 100% disease incidence, and a DI of 65.6. Mock-inoculated controls remained symptomless (Fig. 1l-n). The pathogen was re-isolated and confirmed identical to the inoculum, completing Koch's postulates.

Morphological characterization of representative isolates was conducted using light microscopy and scanning electron microscopy after growth on PDA. Colonies appeared purplish-red, with white to pale pink, cottony aerial mycelium (Fig. 1g). Macroconidia were fusiform to slightly curved, commonly 4–5 septate, measuring $23.9\text{--}31.9 \times 2.9\text{--}4.2 \mu\text{m}$ (n = 50), and displayed distinct surface indentations (Fig. 1h–j). Microconidia were predominantly elliptical with wrinkled surfaces (Fig. 1k). These morphological traits were consistent across representative isolates and supported identification as *F. citri*.

To further confirm taxonomic placement, multilocus sequence analysis was performed using ITS, *TEF1- α* , and *RPB2*. Sequences were compared with reference records in GenBank and showed 99–100% identity to deposited *F. citri* sequences (Additional file 2: Table. S2). Maximum-likelihood phylogenetic reconstruction based on the concatenated loci placed all representative isolates within the *F. citri* clade with strong support (Fig. 1o), clustering closely with the reference strain *F. citri* MAFF 150449. Taken together—field symptomatology, consistent isolation, reproduction of symptoms in inoculation assays, successful re-isolation, and congruent morphological and multilocus phylogenetic evidence—these results establish *F. citri* as the causal agent of alfalfa leaf blight in the surveyed regions of Heilongjiang Province.

Resistance phenotyping of alfalfa cultivars and host responses to *F. citri* infection

Thirty-three alfalfa cultivars were evaluated for resistance to *F. citri* by foliar inoculation, showing substantial variation in disease severity. Based on disease index (DI), cultivars were classified as highly resistant (HR), resistant (R), moderately resistant (MR), or susceptible (S) (Additional file 2: Table. S3). Two cultivars were HR (6.1%; Zhongmu 3, DI = 11.1; Instinct, DI = 13.9), 18 were R (54.5%; DI = 16.7–27.8), 11 were MR (33.3%; DI = 30.6–50.0), and two were S (6.1%; Juneng 551, DI = 59.8; Juneng 7, DI = 66.7).

For downstream analyses, the highly resistant cultivar ZM and the susceptible cultivar JN were selected. Following inoculation, ZM displayed limited blight symptoms, whereas JN developed severe chlorosis and necrosis by 7 dpi (Fig. 1b, Fig. 2a–d). Histopathology showed that ZM maintained more intact tissue organization, while JN exhibited pronounced cellular disruption (Fig. 2a–d). Quantitative analysis revealed stronger structural integrity in ZM: the xylem-positive area increased modestly after infection (0.10%–0.45%) compared to JN (0.28%–2.38%), and the cell wall positive rate remained higher in ZM (16.51%–10.30%) than in JN (13.98%–9.85%) (Additional file 1: Fig. S2, Additional file 2: Table. S4).

At the molecular level, six defense genes (APX, WRKY33, PAL, PR1, PR4, NPR1) exhibited genotype-dependent expression. JN showed strong induction of APX, WRKY33, PR1, PR4, and NPR1 at 3 and 7 dpi, whereas ZM displayed comparatively weaker induction of these genes but pronounced activation of PAL (52.61-fold at 3 dpi, 64.33-fold at 7 dpi) (Fig. 2e–j). Correspondingly, physiological indices (SOD, POD, CAT, MDA, PRO, and soluble protein) changed significantly in a genotype-dependent manner, consistent with the differential transcriptional responses (Fig. 2k–p).

Differences in epiphytic and endophytic bacterial communities between resistant and susceptible alfalfa cultivars after *F. citri* infection

The diversity and composition of epiphytic and endophytic bacterial communities differed between resistant and susceptible alfalfa cultivars following *F. citri* infection (Fig. 3, Additional file 2: Table. S5–6). In the epiphytic compartment, Shannon diversity was significantly reduced in the resistant cultivar after infection (ZM vs. ZMF, $P < 0.001$), whereas no significant change was detected in the susceptible cultivar (JN vs. JNF) (Fig. 1c, Fig. 3a). In contrast, endophytic bacterial Shannon diversity remained relatively stable after infection in both cultivars, with no significant differences observed between infected and non-infected plants (Fig. 3g).

Principal coordinate analysis (PCoA) further revealed clear compositional separation among the four sample groups in both epiphytic and endophytic bacterial communities. For epiphytic bacteria, PCoA1 and PCoA2 explained 53.08% and 35.34% of the variation, respectively, and samples clustered according to host genotype and infection status (Fig. 3b). A similar pattern was observed for endophytic bacteria, where PCoA1 and PCoA2 explained 53.95% and 20.62% of the variation, indicating that both cultivar background and *F. citri* infection contributed to bacterial community reassembly (Fig. 3h).

Differential taxonomic analysis showed that infection-induced shifts were more pronounced in Proteobacteria, Firmicutes, and Bacteroidota, although the magnitude and direction of change differed between host genotypes and compartments. In the epiphytic community of the resistant cultivar,

Pantoea and *Pseudomonas* were enriched after infection, whereas several genera including *Paenibacillus*, *Nocardioides*, *Lawsonella*, *Paracoccus*, *Bacillus*, *Acinetobacter*, and *Pseudarthrobacter* were depleted. In the susceptible cultivar, infection was associated with enrichment of *Xanthomonas*, *Pseudomonas*, *Paenibacillus*, and *Pantoea*, accompanied by depletion of several other genera, including *Pelomonas*, *Serratia*, *Novosphingobium*, *Sphingomonas*, *Nocardioides*, *Acidibacter*, and *Bacillus* (Fig. 3c-f, Additional file 2: Table. S7-8). Similar genotype-dependent shifts were observed in the endophytic compartment. Notably, *Pantoea* and *Pseudomonas* were also enriched in infected resistant plants, whereas the susceptible cultivar showed enrichment of *Xanthomonas*, *Pseudomonas*, *Paenibacillus*, and *Pantoea* (Fig. 3i-l, Additional file 2: Table. S9-10). Together, these results indicate that *F. citri* infection reshaped both epiphytic and endophytic bacterial communities, but the direction and extent of community reassembly differed markedly between resistant and susceptible hosts (Additional file 1: Fig. S3, Additional file 2: Table. S11).

Co-occurrence network differences between resistant and susceptible cultivars

Bacterial co-occurrence networks differed between resistant and susceptible cultivars and were further reshaped after inoculation (Fig. 4, Additional file 2: Table. S12). This divergence was observed in both epiphytic and endophytic communities, but was more pronounced in the endophytic compartment. Overall, endophytic networks were denser than epiphytic networks, with 324–426 edges and average degree values of 3.812–10.390, compared with 84–275 edges and 3.360–9.016 in epiphytic networks. Before inoculation, the susceptible cultivar formed the most connected epiphytic network (275 edges; average degree, 9.016), whereas the resistant cultivar showed lower connectivity (168 edges). After inoculation, epiphytic network complexity decreased in both cultivars and was most strongly reduced in the susceptible cultivar, which retained only 84 edges and an average degree of 3.360. In contrast, endophytic network complexity increased after inoculation in the resistant cultivar, with the resistant post-inoculation group showing the highest connectivity overall (426 edges; average degree, 10.390). Positive correlations predominated across all groups. Together, these results indicate that resistant and susceptible cultivars harbored distinct bacterial interaction patterns and that inoculation induced stronger network reorganization in endophytic than in epiphytic communities.

Functional reprogramming of the resistant cultivar-associated microbiota after inoculation

Inoculation induced clear functional shifts in the microbiota of the resistant cultivar, with stronger predicted changes in the epiphytic than in the endophytic compartment (Fig. 5a, b). In the epiphytic microbiota, pathways related to indole alkaloid biosynthesis ($\log_2FC = 2.91$), lipoarabinomannan biosynthesis ($\log_2FC = 2.35$), lipid metabolism ($\log_2FC = 1.66$), and biosynthesis of various secondary metabolites ($\log_2FC = 1.23$) were significantly enriched after inoculation. In the endophytic microbiota,

several pathways, including *Vibrio cholerae* infection, calcium signaling, monoterpene biosynthesis, and flavonoid-related biosynthesis, also increased in relative abundance, although these changes were not significant after multiple-testing correction. Together, these results indicate that inoculation was associated with more pronounced predicted functional reprogramming in the epiphytic than in the endophytic microbiota of the resistant cultivar.

Isolation, antagonistic activity, and genomic features of strain MX28

A bacterial strain, MX28, was subsequently isolated and identified as *Pantoea agglomerans* based on 16S rRNA phylogeny and whole-genome ANI analysis (Fig. 1d, Fig. 5c, Additional file 1: Fig. S4, Additional file 2: Table. S13). MX28 showed broad antagonistic activity against multiple *Fusarium* species, with inhibition rates ranging from 36.77% to 69.08%, and the strongest activity observed against *F. citri* (69.08%) (Fig. 5d, e, Additional file 2: Table. S14). The genome of MX28 was 4,015,914 bp in size, with a GC content of 55.54%, and contained 3,685 predicted coding sequences, 7 rRNA genes, 69 tRNA genes, and 1 ncRNA (Fig. 5f, Additional file 2: Table. S15-16). Multiple biosynthetic gene clusters were identified, including aryl polyene-, RiPP-, terpene-, and metallophore-associated clusters, with some showing high similarity to known aryl polyene and frederiksenibactin loci (Fig. 5g, Additional file 2: Table. S17). Consistent with these features, functional annotation indicated broad representation of genes involved in metabolism, membrane transport, signal transduction, and secondary metabolism, supporting the antagonistic and adaptive potential of MX28 (Fig. 5h, Additional file 2: Table. S18-19).

Remodeling of non-volatile metabolites and antifungal activity of maculosin

Non-volatile metabolite profiling revealed clear separation among the P, F, and FP groups, with PC1 and PC2 explaining 41.6% and 34.1% of the variance, respectively, and the FP group clearly diverging from both P and F (Fig. 1e, Fig. 6a, Additional file 2: Table. S20). Comparison of P and FP identified 600 differential metabolites ($VIP > 1$, $p < 0.05$), including 239 enriched and 361 depleted metabolites in FP (Fig. 6b, Additional file 1: Fig. S5, Additional file 2: Table. S21). Among the enriched metabolites, malonic acid, cis-acetylacrylic, maculosin, N-acetylvanilalanine, and choline were the most prominent, whereas N-formylmethionine, guanidosuccinic acid, Pro-Tyr, prolyl-histidine, and 3-oxododecanoic acid were among the most depleted (Fig. 6c). These metabolites were mainly assigned to pathways related to penicillin and cephalosporin biosynthesis, degradation of flavonoids, 2-oxocarboxylic acid metabolism, phenylalanine/tyrosine/tryptophan biosynthesis, and ubiquinone and other terpenoid-quinone biosynthesis (Fig. 6d). Notably, maculosin accumulated to markedly higher levels in FP than in either P or F, whereas no significant difference was detected between P and F (Fig. 6e). Maculosin also inhibited *Fusarium* mycelial growth in a concentration-dependent manner across $1-128 \mu\text{g ml}^{-1}$, reaching 86.7% inhibition at $128 \mu\text{g ml}^{-1}$ (Fig. 6f). Scanning electron microscopy showed that untreated hyphae were intact and smooth, whereas maculosin-treated hyphae exhibited roughened surfaces, shrinkage,

collapse, fracture, and partial dissolution (Fig. 6f). Consistent with this, the EC_{50} of maculosin ($12.80 \mu\text{g ml}^{-1}$) was lower than that of hymexazol ($24.94 \mu\text{g ml}^{-1}$) (Fig. 6h), indicating stronger antifungal activity under the tested conditions.

Biocontrol effects of *P. agglomerans* MX28 and associated defense responses

In pot experiments, plants inoculated with *F. citri* alone developed severe chlorosis, wilting, lodging, and overall decline, whereas plants treated with MX28 alone or maculosin alone remained healthy (Fig. 6i). Disease symptoms were markedly reduced in both the *Fusarium* + MX28 and *Fusarium* + maculosin treatments. Disease index measurements supported these observations: the *Fusarium*-only treatment increased from 19 at 7 d to 57 at 10 d and 96 at 14 d, whereas the corresponding values at 14 d were 20 for *Fusarium* + MX28 and 12.83 for *Fusarium* + maculosin (Fig. 6j). Both combined treatments differed significantly from the *Fusarium*-only treatment, while no significant difference was detected between them.

All treatments induced host defense-related transcription to varying degrees, with the strongest responses generally observed in the *Fusarium* + MX28 treatment (Fig. 6k-n). *PAL* was strongly induced by pathogen infection, whereas *PR1*, *PR4*, and *WRKY33* reached their highest expression levels in the *Fusarium* + MX28 treatment. Targeted metabolite analysis further confirmed the presence of maculosin in the MX28 metabolome. Genome mining identified an NRPS biosynthetic gene cluster containing *mclA*, a putative maculosin-associated biosynthetic gene, while *tonB* was predicted to be involved in iron acquisition and nutrient competition. Consistent with these predictions, both *tonB* and *mclA* were significantly upregulated, reaching 12.5-fold and 6.1-fold relative to the control, respectively (Fig. 6o, p). Together, these results suggest that disease suppression by MX28 may involve direct antifungal activity mediated by maculosin, enhanced iron competition, and activation of host defense responses.

Discussion

Here, we combined pathogen identification, host phenotyping, microbiome profiling, strain isolation, metabolite characterization, and targeted expression analysis to investigate alfalfa leaf blight caused by *F. citri*. Three main conclusions emerge from this work. First, *F. citri* was consistently identified as the causal agent of alfalfa leaf blight in the surveyed alfalfa-growing regions. Second, host resistance was associated not only with attenuated disease development and preservation of tissue integrity, but also with pronounced restructuring of both epiphytic and endophytic bacterial communities following pathogen challenge. Third, this restructuring yielded a culturable antagonist, *P. agglomerans* MX28, whose disease-suppressive activity was associated with direct antifungal effects, altered metabolite output including maculosin, and enhanced host defense responses. Together, these findings support a microbiome-centered view of disease outcome in which host genotype, pathogen infection, and microbiota function act in concert to shape the alfalfa leaf blight phenotype.

A key starting point of this study was etiological clarification. The repeated isolation of *F. citri* from symptomatic tissues, reproduction of disease symptoms following inoculation, successful re-isolation, and concordant morphological and multilocus phylogenetic evidence together provide strong support for assigning causality to *F. citri*. This point is important not only diagnostically, but also conceptually. In *Fusarium*, morphology alone is often insufficient for confident species delimitation, whereas multilocus approaches incorporating TEF1- α and RPB2 loci substantially improve taxonomic resolution. Establishing a single, well-supported causal agent therefore provides an essential foundation for interpreting downstream host and microbiome responses within a defined pathosystem rather than a mixed-pathogen context^{25,26}.

The comparison between the resistant cultivar ZM and the susceptible cultivar JN further indicates that resistance to *F. citri* reflects qualitatively distinct host response states rather than a simple reduction in visible symptom severity. ZM retained greater tissue organization and cell-wall integrity after inoculation, whereas JN displayed stronger chlorosis, necrosis, and tissue disruption. At the transcriptional level, the most notable feature was the strong induction of PAL in ZM, whereas JN showed greater induction of *APX*, *WRKY33*, *PR1*, *PR4*, and *NPR1*. One plausible interpretation is that the resistant genotype mounted a more effective phenylpropanoid- and barrier-associated response, whereas the stronger induction of several canonical defense markers in the susceptible genotype reflected greater pathogen pressure and tissue damage rather than more effective resistance. This interpretation is consistent with the central role of *PAL* in phenylpropanoid metabolism and broad-spectrum disease resistance, and with the established function of *WRKY33* in defense against necrotrophic fungal pathogens^{27–29}.

A major finding of this study is that *F. citri* infection was accompanied by marked bacterial community reassembly in both the epiphytic and endophytic compartments, with the direction and magnitude of this response depending on host genotype. The phyllosphere is a nutrient-limited and environmentally variable habitat in which microbial assemblages can change rapidly under biotic stress, and increasing evidence suggests that pathogen challenge can both destabilize resident communities and favor the enrichment of taxa with protective potential^{30–32}. In our system, infection of the resistant cultivar was associated with enrichment of *Pantoea* and *Pseudomonas*, whereas the susceptible cultivar showed a distinct pattern of reassembly that also included taxa more often associated with disease-linked states. Although amplicon sequencing does not resolve functional activity at strain level, the selective enrichment of *Pantoea* and *Pseudomonas* in the resistant background is notable, given that both genera include phyllosphere-competent strains with documented antagonistic, competitive, and immune-modulatory activities^{33–35}. The co-occurrence analysis extends this ecological signal by suggesting that resistance was associated not only with taxonomic turnover, but also with altered community organization, particularly in the endophytic compartment. Inoculation reduced epiphytic network complexity in both cultivars, most strongly in the susceptible line, whereas endophytic network complexity increased in the infected resistant cultivar and reached the highest connectivity overall. One possible interpretation is that the resistant host provides a more stable or selectively permissive internal niche in which bacterial assemblages remain organized under pathogen pressure. At the same time, this

conclusion should be treated cautiously. Co-occurrence networks inferred from amplicon data reflect statistical associations rather than demonstrated ecological interactions, and bacteria-only networks do not capture cross-kingdom fungal-bacterial relationships that may be especially relevant in a *Fusarium* pathosystem^{23,36,37}. Accordingly, these results are best interpreted as evidence of differential microbiome organization rather than direct proof of specific interaction mechanisms³⁸. The functional prediction analysis points in the same overall direction, while also defining an important mechanistic boundary of the present study. The stronger predicted functional reprogramming observed in the epiphytic microbiota of the resistant cultivar is biologically plausible, given that leaf-surface communities represent the first microbial interface encountered by foliar pathogens^{32,39}. Enrichment of functions related to secondary metabolism and other stress-associated processes after inoculation is consistent with a microbiome shifting toward a more chemically responsive state²². However, because these inferences were derived from marker gene-based prediction rather than directly measured metagenomic, metatranscriptomic, or metabolomic activity at the community level, they should be regarded as hypothesis-generating rather than functionally validated^{40–42}.

The isolation of *P. agglomerans* MX28 provides a culture-based bridge between sequencing-derived community patterns and an experimentally tractable biocontrol phenotype. This is an important strength of the study, because many microbiome analyses remain correlative, whereas our work progresses from taxonomic enrichment to isolate recovery, genome analysis, metabolite profiling, and disease suppression assays^{43,44}. *Pantoea* spp. are increasingly recognized as context-dependent plant-associated bacteria with substantial biocontrol potential, mediated through antibiosis, resource competition, and induction of plant innate immunity. At the same time, the genus is functionally diverse, making strain-level validation essential. In this context, the broad antagonistic activity of MX28 against multiple *Fusarium* species, together with its genomic potential for secondary metabolism, supports both its ecological relevance and its translational potential⁴⁵.

Among the mechanistic findings, the maculosin result is arguably the most distinctive. We detected maculosin in the MX28 metabolome, observed its enrichment in the confrontation condition, demonstrated concentration-dependent inhibition of *Fusarium* mycelial growth, and visualized marked hyphal deformation following treatment. Taken together, these observations support a direct antifungal contribution of maculosin under the conditions tested. However, the transition from metabolite detection to biosynthetic assignment should remain conservative^{46,47}. Maculosin was originally described as a diketopiperazine phytotoxin from *Alternaria alternata*, and its occurrence in a *Pantoea*-associated antagonistic context is therefore particularly intriguing⁴⁸. Our genome mining and transcriptional data identify mclA as a plausible maculosin-associated biosynthetic candidate, but not yet as a functionally validated determinant. Reverse-genetic or heterologous-expression approaches will be required to establish whether the identified NRPS-associated locus is directly responsible for maculosin production in MX28⁴⁹.

A similar level of caution applies to the proposed role of iron competition. The strong induction of *tonB* is consistent with enhanced nutrient-acquisition capacity, and such a mechanism is biologically credible because iron availability is a major axis of competition during plant infection. Iron withholding and iron scavenging are increasingly recognized as important determinants of plant-pathogen outcomes, and TonB-dependent transport systems are well known to support the uptake of scarce extracellular substrates, including iron complexes, in Gram-negative bacteria^{50,51}. In this context, the upregulation of *tonB* in MX28 is compatible with a competitive fitness mechanism that may complement direct antibiosis. Nevertheless, the present data support a likely contribution rather than a definitive iron-competition mechanism. The most defensible interpretation is therefore that MX28-mediated disease suppression involves at least three partially convergent processes: direct antifungal activity associated with maculosin, nutrient competition consistent with TonB-linked acquisition functions, and enhancement of host defense responses^{34,52}.

The host transcriptional data from the pot experiment further reinforce this integrated model. The strongest overall induction of *PR1*, *PR4*, and *WRKY33* in the *Fusarium* + MX28 treatment suggests that MX28 does more than suppress the pathogen externally; it also appears to potentiate host defense signaling^{53,54}. This interpretation is consistent with the broader literature on phyllosphere-associated biocontrol, in which beneficial bacteria can couple ecological suppression of pathogens with immune activation in the host⁵⁵. The comparison between MX28 and purified maculosin is particularly informative in this regard. Although maculosin alone reproduced much of the disease-suppressive phenotype, MX28 elicited stronger defense-related transcriptional responses, suggesting that the living bacterium likely contributes additional activities beyond the purified metabolite itself. These may include colonization-dependent signaling, nutrient sequestration, or production of other bioactive metabolites encoded in the MX28 genome⁵⁶.

Overall, our findings support a model in which host genotype shapes microbiome reassembly after *F. citri* challenge, this reassembly is associated with enrichment of potentially beneficial taxa such as *Pantoea* and *Pseudomonas*, and at least one recruited strain, MX28, contributes to disease suppression through a combination of antifungal chemistry, probable nutrient competition, and host defense activation. This framework moves beyond a purely pathogen-centric view of alfalfa leaf blight and instead places disease development within a dynamic host-microbiome-pathogen system. Several next steps now become clear. Future work should quantify absolute bacterial and fungal abundances, move from predicted to directly measured microbiome functions using metagenomic or transcriptomic approaches, incorporate cross-kingdom network analysis, and test the causal roles of *mclA* and *tonB* through reverse genetics. Even with these current limitations, the present study provides a strong foundation for microbiome-assisted management of alfalfa leaf blight and expands the mechanistic basis for interpreting disease suppression in foliar pathosystems.

Materials and Methods

Disease survey and sample collection

Field surveys of alfalfa leaf blight were conducted from late July to early August 2024 in major alfalfa-producing regions of Heilongjiang Province, China. A total of 250 symptomatic samples were collected from 10 sites across four cities, including Harbin, Daqing, Qiqihar, and Zhaodong. The geographic distribution of the sampling sites is shown in Additional file 1: Fig. S1. Leaves displaying typical blight symptoms were collected in sterile bags and transported to the laboratory for pathogen isolation.

Fungal isolation, purification and preservation

Leaf tissues were excised from the margins of symptomatic lesions and cut into approximately 5 × 5 mm pieces. Tissue segments were surface-sterilized in 75% ethanol for 30 s, immersed in 1% sodium hypochlorite for 1 min, rinsed three times in sterile distilled water, and dried on sterile filter paper. Segments were placed on potato dextrose agar (PDA) and incubated at 25 °C in the dark for 3–5 d. Emerging colonies were purified by hyphal tip transfer. In total, 61 *F. citri* isolates were obtained and designated MYK1–MYK61. Purified isolates were maintained in 20% glycerol at – 80 °C.

Pathogenicity assays and Koch's postulates

Pathogenicity assays were performed on the alfalfa cultivar Dongnong 1. Isolate MYK1 was grown on PDA for 7 d, and conidia were harvested and suspended in sterile water containing 0.05% Tween 20 to a final concentration of 1×10^6 conidia mL⁻¹. Plants were spray-inoculated with 5 mL of conidial suspension per plant. Control plants were sprayed with sterile water only. After inoculation, plants were enclosed to maintain high humidity for 24 h and then incubated at 25°C under a 16 h light/8 h dark photoperiod. Each treatment comprised six plants with five biological replicates. Disease severity was assessed at 7 dpi using a disease index (DI) scale as follows: 0, no visible symptoms; 1, 1–10% leaf area affected; 2, 11–25% leaf area affected; 3, 26–50% leaf area affected; 4, 51–75% leaf area affected; and 5, > 75% leaf area affected or leaf death. The pathogen was re-isolated from symptomatic leaves and re-identified based on morphology and molecular characteristics to complete Koch's postulates.

Morphological characterization

Representative isolates were cultured on PDA at 25 °C for 7 d, and colony characteristics, including pigmentation, aerial mycelium and colony texture, were recorded. Micromorphological observations were performed using a SOPTOP EX21 light microscope. For scanning electron microscopy (SEM), fungal samples were fixed in 2.5% glutaraldehyde, dehydrated through a graded ethanol series, subjected to critical-point drying, sputter-coated with gold, and examined with a SU8010 field-emission scanning electron microscope at an accelerating voltage of 5 kV. For each isolate, 50 macroconidia and 50 microconidia were measured.

Molecular identification and phylogenetic analysis

Genomic DNA was extracted from representative isolates using a NuClean Plant Genomic DNA Kit (Kangwei Century Biotechnology Co., Jiangsu, China) following a CTAB-based procedure. Three loci, including the internal transcribed spacer (ITS), translation elongation factor 1- α (*TEF1- α*) and RNA polymerase II second largest subunit (*RPB2*), were amplified using primer pairs listed in Additional file 2: Table. S1. PCR was performed in a 25 μ L reaction volume containing 12.5 μ L 2 $\times$ Taq PCR Master Mix (CWBIO, Beijing, China), 1 μ L of each primer (10 μ M), 1 μ L template DNA and 9.5 μ L nuclease-free water. The amplification conditions were 94 $^{\circ}$ C for 5 min; 35 cycles of 94 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 1 min; followed by 72 $^{\circ}$ C for 10 min. Amplicons were subjected to Sanger sequencing, and the resulting sequences were queried against the GenBank database using BLAST. Multilocus phylogenetic analysis was conducted in MEGA using the maximum-likelihood method with 1,000 bootstrap replicates

57

Evaluation of cultivar resistance

Thirty-three alfalfa cultivars were evaluated for resistance to *F. citri*. Inoculation conditions were identical to those used in the pathogenicity assays. For each cultivar, six plants were included per replicate, with five biological replicates. Disease severity was recorded at 7 dpi, and DI was calculated based on the scoring system described above. Cultivars were classified as highly resistant (HR, DI $\leq$ 15%), resistant (R, 16% < DI $\leq$ 30%), moderately resistant (MR, 31% < DI $\leq$ 50%), susceptible (S, 51% < DI $\leq$ 70%) or highly susceptible (HS, DI > 71%). Based on these results, Zhongmu 3 (ZM) and Juneng 7 (JN) were selected as representative resistant and susceptible cultivars, respectively, for subsequent analyses (Additional file 2: Table. S3).

Histopathological observation

Leaf samples collected at 0 and 7 dpi were processed for paraffin sectioning. Tissues were fixed in FAA fixative prepared from acetic acid, formalin and 50–70% ethanol at a ratio of 1:1:18 (v/v), dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, sectioned, and stained with safranin and fast green according to conventional histological procedures. Image acquisition and quantitative analysis were performed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). Xylem-positive area and cell wall positive rate were quantified from stained sections based on standard threshold-based image analysis.

RNA extraction and quantitative real-time PCR

For analysis of host defense-related gene expression during infection, alfalfa leaves were sampled at 0, 3 and 7 dpi. Total RNA was extracted using an Ultrapure RNA Kit (Kangwei Century Biotechnology Co., Jiangsu, China). First-strand cDNA was synthesized using HiScript II Q RT SuperMix for qPCR (Vazyme Biotechnology Co., Nanjing, China). Quantitative real-time PCR (qRT-PCR) was performed using the gene-specific primers listed in Additional file 2: Table. S2. Each 20 μ L reaction contained 10 μ L 2 $\times$ SYBR Green qPCR Master Mix, 0.8 μ L of each primer (10 μ M), 2 μ L diluted cDNA and 6.4 μ L nuclease-free water. The cycling conditions were 95 $^{\circ}$ C for 30 s, followed by 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 30 s. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method with actin as the reference gene

⁵⁸. Five biological replicates were analysed for each treatment. For the pot experiments, expression of the host defense genes *PAL*, *PR1*, *PR4* and *WRKY33* was analysed at 14 d using the same procedures. Expression of the bacterial genes *mclA* and *tonB* was also determined from treated leaf samples using gene-specific primers listed in Supplementary Table S1.

Determination of physiological indices

Leaf tissues were collected for determination of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), malondialdehyde (MDA), proline (PRO) and soluble protein. All measurements were performed using commercial assay kits from Suzhou Keming Biotechnology Co., Ltd., following the manufacturer's instructions. Three biological replicates were included for each assay.

Epiphytic and endophytic bacterial community analysis

Leaves from four groups, ZM, ZMF, JN and JNF, were collected for bacterial community analysis. Each group comprised five biological replicates, and each replicate consisted of pooled leaves from five plants. For analysis of epiphytic bacteria, leaf-associated microbes were detached by washing leaves in phosphate-buffered saline (PBS) followed by shaking. The resulting suspension was centrifuged at 5,000 rpm to collect microbial cells. For endophytic bacterial analysis, leaf surfaces were sterilized and the tissues were subsequently homogenized for DNA extraction.

Total DNA was extracted using the CretMag Power Soil DNA Kit. DNA quality and concentration were assessed by agarose gel electrophoresis and NanoDrop 2000. The V3–V4 region of the bacterial 16S rDNA gene was amplified using primer pairs listed in Supplementary Table S5. PCR products from the same sample were pooled, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), quantified using a Quantus Fluorometer, and used for library construction with the VAHTS Universal Plus DNA Library Prep Kit for MGI V2. Libraries passing quality control were sequenced on the MGI platform in paired-end mode (PE250).

Raw reads were demultiplexed according to barcode and primer information, quality filtered, merged and chimera filtered within the microbiome analysis workflow. ASVs were generated in QIIME 2 using the denoising pipeline provided in the report ⁵⁹. Alpha-diversity and beta-diversity analyses were conducted based on the ASV abundance table. Community structure was visualized using principal coordinate analysis (PCoA), principal component analysis (PCA) and non-metric multidimensional scaling (NMDS). Differential taxa and between-group differences were analysed using the statistical modules implemented in the microbiome workflow. Functional prediction was performed using PICRUST2.

Isolation, identification and antagonistic activity of strain MX28

Strain MX28 was isolated from the epiphytic bacterial community of infected resistant plants (ZMF) on LB agar using routine bacterial isolation procedures. Preliminary identification was conducted by amplification of the 16S rRNA gene using primers 27F and 1492R. Antagonistic activity against *F. citri*

isolate MYK1 was evaluated by dual-culture assay. A 5-mm mycelial plug was placed at the centre of a PDA plate, and the bacterial strain was inoculated at four equidistant points near the plate margin. Plates inoculated with the fungus alone served as controls. All plates were incubated in the dark at 26 °C for 7 d. The inhibition rate was calculated as $100 \times [(R1 - R2)/R1]$, where R1 is the radial growth of the fungus in the control and R2 is the radial growth in the dual-culture assay. Each treatment was performed in triplicate.

Whole-genome sequencing and genomic analysis of MX28

Genomic DNA of MX28 was extracted using the MagPure Bacterial DNA Kit. Sequencing libraries were prepared with the Hieff NGS MaxUp II DNA Library Prep Kit for Illumina and sequenced on the MGI DNBSeg-T7 or Illumina NovaSeq 6000 platform using PE150 reads, yielding approximately 1 Gb of clean data. Raw reads were quality filtered using fastp, assembled using SPAdes, gap-filled using GapFiller, and polished using Pilon/PrInSeS-G according to the genome analysis report. Gene prediction and annotation were performed using Prokka, while similarity searches against the NCBI NT database were conducted using BLAST+. Average nucleotide identity (ANI) analysis was performed against reference genomes to support species assignment⁶⁰. Biosynthetic gene clusters were predicted using antiSMASH⁶¹.

Non-volatile metabolomics analysis

Three treatment groups were included in the non-volatile metabolomics experiment: *P. agglomerans* alone (P), *F. citri* alone (F), and confrontation co-culture of *P. agglomerans* and *F. citri* (FP). Each group included three biological replicates, and samples were collected after 7 d of incubation. According to the metabolomics workflow, 40 ± 1 mg biomass was extracted with 400 μ L 80% methanol/water containing internal standard. Samples were homogenized with steel beads, subjected to repeated freeze–thaw cycles in liquid nitrogen, and centrifuged at 12,000 rpm at 4 °C. Supernatants were collected for LC–MS analysis.

Metabolite profiling was performed using a Vanquish UHPLC system coupled to a Q Exactive HF-X mass spectrometer. Chromatographic separation was achieved on a Waters ACQUITY Premier HSS T3 column (1.8 μ m, 2.1 $\times$ 100 mm) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The column temperature was maintained at 40°C, the flow rate was 0.4 mL min⁻¹ and the injection volume was 3 μ L. Mass spectra were acquired in both positive and negative ionization modes over an m/z range of 70–1000. Raw data were converted to mzML format using ProteoWizard and processed in XCMS for peak detection, retention-time correction and alignment. Missing values were imputed according to the report workflow, and peak areas were corrected using support vector regression. Differential metabolites were screened using the criteria applied in this study (VIP > 1 and $P < 0.05$), and KEGG was used for pathway annotation and enrichment analysis.

Antifungal activity of maculosin

Maculosin standard (purity 99.0%) was used to evaluate antifungal activity against *F. citri*. Maculosin was incorporated into PDA at final concentrations of 1, 2, 4, 8, 16, 32, 64 and 128 $\mu\text{g mL}^{-1}$. Hymexazol was included as a chemical reference at the same concentration series. A mycelial plug of *F. citri* was placed at the centre of each amended plate, and fungal growth was measured after incubation. The half-maximal effective concentration (EC_{50}) was estimated from dose–response curves. For SEM observation, hyphae from treated and untreated plates were processed using the same fixation and dehydration procedures described above.

Pot experiments for biocontrol evaluation

Pot experiments were conducted using the susceptible alfalfa cultivar Juneng 7 (JN). Plants were grown in soil in 15-cm pots under greenhouse conditions at 26 °C and relative humidity above 85%. Six treatments were established: water control, *F. citri* alone, MX28 alone, *F. citri* + MX28, maculosin alone, and *F. citri* + maculosin. MX28 was applied by spraying a bacterial suspension at $1 \times 10^8 \text{ CFU mL}^{-1}$, maculosin was applied at $12.8 \mu\text{g mL}^{-1}$, and *F. citri* was inoculated at $1 \times 10^6 \text{ conidia mL}^{-1}$. In the combined treatment, MX28 was applied 24 h before fungal inoculation. Each treatment included six plants with five biological replicates. Disease severity was assessed at 7, 10 and 14 d after inoculation using the same DI scale described above.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism and R. Differences between two groups were assessed using t-test where appropriate. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. For microbiome beta-diversity analyses, permutational multivariate analysis of variance (PERMANOVA) was used to assess community differences based on distance matrices. Where multiple testing was involved, P values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method. Differences were considered statistically significant at $P < 0.05$ or $\text{FDR} < 0.05$.

Declarations

Data availability

All data supporting the findings of this study are available within the Article and its Supplementary Information. Raw 16S rDNA sequencing data have been deposited in the NCBI BioProject database under accession number PRJNA1447158. Raw metabolomics data have been deposited in the OMIX database under accession number OMIX016038. The 16S rRNA gene sequence of *Pantoea agglomerans* MX28 has been deposited in NCBI under accession number PZ240735.

Code availability

No custom code was generated or used in this study.

Acknowledgements

This work was supported by the Science and Technology Innovation 2030 Major Project (2022ZD040120401).

Author contributions

G.C. conceived and supervised the study, acquired funding, and wrote the original draft of the manuscript with subsequent review and editing. M.T., B.W., Q.Z. and X.Z. performed the experiments and developed the methodology. X.K., Y.X., Q.R. and W.Z. conducted field sampling and contributed to data curation and formal analysis. M.M. contributed to validation and manuscript review and editing. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

References

1. Abbas, A. *et al.* Root rot a silent alfalfa killer in China: Distribution, fungal, and oomycete pathogens, impact of climatic factors and its management. *Frontiers in Microbiology* **13**, 28, doi:10.3389/fmicb.2022.961794 (2022).
2. Zhou, Z. *et al.* Research on drought stress in *Medicago sativa* L. from 1998 to 2023: a bibliometric analysis. *Frontiers in Plant Science* **15**, 16 (2024).
3. Pellegrino, E., Nuti, M. & Ercoli, L. Multiple arbuscular mycorrhizal fungal consortia enhance yield and fatty acids of *Medicago sativa*: a two-year field study on agronomic traits and tracing of fungal persistence. *Frontiers in plant science* **13**, 814401 (2022).
4. Koptileuov, B. *et al.* The Influence of the Timing of Haymaking on the Yield and Quality of Alfalfa Feed (*Medicago sativa* L.) in the Conditions of the Kazakhstan Aral Sea Region. *Preprints*, doi:10.20944/preprints202305.0831.v1 (2023).
5. Jones, R. A. C. & Naidu, R. A. in *Annual Review of Virology* Vol. 6 *Annual Review of Virology* (eds L. Enquist, D. DiMaio, & T. Demody) 387-409 (Annual Reviews, 2019).
6. Wang, R., Liu, J., Jiang, W., Ji, P. & Li, Y. Metabolomics and microbiomics reveal impacts of rhizosphere metabolites on alfalfa continuous cropping. *Frontiers in Microbiology* **13**, 833968 (2022).
7. Zhu, Y. *et al.* Different Disease Levels Reveal Kiwifruit Brown Spot Impacts on Fruit Yield and Quality. *Journal of Fungi* **11**, 593 (2025).
8. Bahadur, A. in *Fusarium-an overview of the genus* (IntechOpen, 2021).
9. Ekwomadu, T. I. & Mwanza, M. *Fusarium* fungi pathogens, identification, adverse effects, disease management, and global food security: A review of the latest research. *Agriculture* **13**, 1810 (2023).

10. Xia, J., Sandoval-Denis, M., Crous, P. W., Zhang, X. & Lombard, L. Numbers to names-restyling the *Fusarium incarnatum-equiseti* species complex. *Persoonia-Molecular Phylogeny Evolution of Fungi* **43**, 186-221 (2019).
11. Jiahui, L. et al. Effects of bacterial wilt on community composition and diversity of culturable endophytic fungi in *Alpinia galanga*. *bioRxiv* **23**, 625029 (2024).
12. O'Donnell, K. et al. DNA sequence-based identification of *Fusarium*: A work in progress. *Plant disease* **106**, 1597-1609 (2022).
13. Wang, J.-H., Ndoeye, M., Zhang, J.-B., Li, H.-P. & Liao, Y.-C. Population structure and genetic diversity of the *Fusarium graminearum* species complex. *Toxins* **3**, 1020-1037 (2011).
14. Noman, M. et al. Bacterial extracellular biomolecules-derived multimodal manganese nanoparticles control watermelon *Fusarium* wilt by dysregulating fusaric acid biosynthesis pathway and precise tuning of rhizosphere metabolome. *Journal of Nanobiotechnology* **23**, 452 (2025).
15. Jia, H. et al. Genome editing of the disease susceptibility gene Cs LOB 1 in citrus confers resistance to citrus canker. *Plant biotechnology journal* **15**, 817-823 (2017).
16. Nawrocka, J., Szymczak, K., Skwarek-Fadecka, M. & Małolepsza, U. Toward the analysis of volatile organic compounds from tomato plants (*Solanum lycopersicum* L.) treated with *Trichoderma virens* or/and *Botrytis cinerea*. *Cells* **12**, 1271 (2023).
17. Gayathri, M. et al. Genomic configuration of *Bacillus subtilis* (NMB01) unveils its antiviral activity against Orthotospovirus arachnecrosis infecting tomato. *Frontiers in plant science* **16**, 1517157 (2025).
18. Alkan, M. et al. Monitoring of host suitability and defense-related genes in wheat to *Bipolaris sorokiniana*. *Journal of Fungi* **8**, 149 (2022).
19. Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T. & Singh, B. K. J. N. r. m. Plant–microbiome interactions: from community assembly to plant health. **18**, 607-621 (2020).
20. Du, Y., Han, X. & Tsuda, K. J. J. o. G. P. P. Microbiome-mediated plant disease resistance: recent advances and future directions. **91**, 1-17 (2025).
21. Vogel, C. M., Potthoff, D. B., Schäfer, M., Barandun, N. & Vorholt, J. A. J. N. m. Protective role of the *Arabidopsis* leaf microbiota against a bacterial pathogen. **6**, 1537-1548 (2021).
22. Li, P.-D. et al. The phyllosphere microbiome shifts toward combating melanose pathogen. **10**, 56 (2022).
23. Kajihara, K. T. & Hynson, N. A. J. M. Networks as tools for defining emergent properties of microbiomes and their stability. **12**, 184 (2024).
24. Pang, Z., Xu, J. & Yi, C. J. F. i. P. S. Linking Plant Secondary Metabolites and Plant Microbiomes: A Review. **The Plant Holobiont Volume II: Impacts of the Rhizosphere on Plant Health** (2021).
25. O'Donnell, K. et al. DNA sequence-based identification of *Fusarium*: A work in progress. **106**, 1597-1609 (2022).

26. Bhunjun, C. S., Phillips, A. J., Jayawardena, R. S., Promputtha, I. & Hyde, K. D. J. P. Importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's postulates. **10**, 1096 (2021).
27. Wang, R., Wang, G.-L. & Ning, Y. J. T. i. p. s. PALs: emerging key players in broad-spectrum disease resistance. **24**, 785-787 (2019).
28. Molina, A. *et al.* Plant cell wall-mediated disease resistance: Current understanding and future perspectives. **17**, 699-724 (2024).
29. Chen, Y. & Zhang, J. J. G. Multiple functions and regulatory networks of WRKY33 and its orthologs. **931**, 148899 (2024).
30. De Mandal, S. & Jeon, J. J. P. Phyllosphere microbiome in plant health and disease. **12**, 3481 (2023).
31. Vacher, C. *et al.* The phyllosphere: microbial jungle at the plant–climate interface. **47**, 1-24 (2016).
32. Perreault, R. & Laforest-Lapointe, I. J. T. I. j. Plant-microbe interactions in the phyllosphere: facing challenges of the anthropocene. **16**, 339-345 (2022).
33. Shalev, O. *et al.* Commensal *Pseudomonas* strains facilitate protective response against pathogens in the host plant. **6**, 383-396 (2022).
34. Duchateau, S., Crouzet, J., Dorey, S. & Aziz, A. J. B. C. The plant-associated *Pantoea* spp. as biocontrol agents: Mechanisms and diversity of bacteria-produced metabolites as a prospective tool for plant protection. **188**, 105441 (2024).
35. Pfeilmeier, S. *et al.* Leaf microbiome dysbiosis triggered by T2SS-dependent enzyme secretion from opportunistic *Xanthomonas* pathogens. **9**, 136-149 (2024).
36. Faust, K. J. T. I. j. Open challenges for microbial network construction and analysis. **15**, 3111-3118 (2021).
37. Röttgers, L. & Faust, K. J. F. m. r. From hairballs to hypotheses—biological insights from microbial networks. **42**, 761-780 (2018).
38. Lee, K. K., Kim, H. & Lee, Y.-H. J. F. i. m. Cross-kingdom co-occurrence networks in the plant microbiome: Importance and ecological interpretations. **13**, 953300 (2022).
39. Zhan, C., Matsumoto, H., Liu, Y. & Wang, M. J. N. F. Pathways to engineering the phyllosphere microbiome for sustainable crop production. **3**, 997-1004 (2022).
40. Toole, D. R., Zhao, J., Martens-Habbena, W. & Strauss, S. L. J. A. S. E. Bacterial functional prediction tools detect but underestimate metabolic diversity compared to shotgun metagenomics in southwest Florida soils. **168**, 104129 (2021).
41. Sun, S., Jones, R. B. & Fodor, A. A. J. M. Inference-based accuracy of metagenome prediction tools varies across sample types and functional categories. **8**, 46 (2020).
42. Douglas, G. M. *et al.* PICRUSt2 for prediction of metagenome functions. **38**, 685-688 (2020).
43. Lorenzi, A. S., Bonatelli, M. L., Chia, M. A., Peressim, L. & Quecine, M. C. J. M. Opposite sides of *Pantoea* agglomerans and its associated commercial outlook. **10**, 2072 (2022).

44. Dahiya, P., Kumar, P., Rani, S., Dang, A. S. & Suneja, P. J. C. M. Comparative genomic and functional analyses for insights into *Pantoea* agglomerans strains adaptability in diverse ecological niches. **81**, 254 (2024).
45. Smits, T. H., Duffy, B., Blom, J., Ishimaru, C. A. & Stockwell, V. O. J. A. o. m. Pantocin A, a peptide-derived antibiotic involved in biological control by plant-associated *Pantoea* species. **201**, 713-722 (2019).
46. Kirk, A., Davidson, E. & Stavrinos, J. J. M. R. The expanding antimicrobial diversity of the genus *Pantoea*. **289** (2024).
47. Scherlach, K. & Hertweck, C. J. N. C. Mining and unearthing hidden biosynthetic potential.
48. Stierle, A. C., Cardellina, J. H. & Strobel, G. A. J. P. o. t. N. A. o. S. Maculosin, a host-specific phytotoxin for spotted knapweed from *Alternaria alternata*. **85**, 8008-8011 (1988).
49. Walters, A. M., Smith, D. D. N., John, S. & Mark, G. J. P. O. Identification of a *Pantoea* Biosynthetic Cluster That Directs the Synthesis of an Antimicrobial Natural Product. **9**, e96208 (2014).
50. Krewulak, K. D., Vogel, H. J. J. B. & Biology, C. TonB or not TonB: is that the question? **89**, 87-97 (2011).
51. Sun, J., Xiao, S. & Xue, C. J. P. R. The tug-of-war on iron between plant and pathogen. **5**, 61 (2023).
52. Wang, J. *et al.* Application of TonB-dependent transporters in vaccine development of gram-negative bacteria. **10**, 589115 (2021).
53. Javed, T. & Gao, S.-J. J. T. i. G. WRKY transcription factors in plant defense. **39**, 787-801 (2023).
54. Yu, Y. *et al.* Induced systemic resistance for improving plant immunity by beneficial microbes. **11**, 386 (2022).
55. Legein, M. *et al.* Modes of action of microbial biocontrol in the phyllosphere. **11**, 1619 (2020).
56. Duchateau, S., Crouzet, J., Dorey, S. & Aziz, A. J. B. C. The plant-associated *Pantoea* spp. as biocontrol agents: Mechanisms and diversity of bacteria-produced metabolites as a prospective tool for plant protection. **188**, 11 (2023).
57. Tamura, K., Stecher, G., Kumar, S. J. M. b. & evolution. MEGA11: molecular evolutionary genetics analysis version 11. **38**, 3022-3027 (2021).
58. Bustin, S. A. *et al.* MIQE 2.0: revision of the minimum information for publication of quantitative real-time PCR experiments guidelines. **71**, 634-651 (2025).
59. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. **37**, 852-857 (2019).
60. Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T. & Aluru, S. J. N. c. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. **9**, 5114 (2018).
61. Blin, K. *et al.* antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. **51**, W46-W50 (2023).

Figures

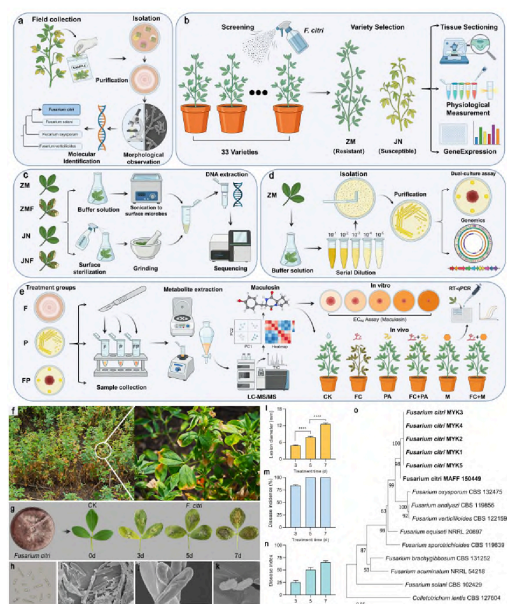


Figure 1. Disease characterization of alfalfa leaf blight and identification of a resistance-associated antagonist. (a–e). Schematic overview of the study workflow, including pathogen isolation and identification from symptomatic alfalfa, screening of 33 alfalfa varieties to select the resistant cultivar Zhongmu 3 (ZM) and the susceptible cultivar Juneng 7 (JN), sampling of epiphytic and endophytic bacterial communities for sequencing, isolation

and antagonism screening of culturable bacteria from ZM leaves, and subsequent metabolite, genomic, and host response analyses. ZMF, diseased leaves of ZM; JNF, diseased leaves of JN; F, *Fusarium citri*; P, *Pantoea agglomerans*; FP, combined treatment of *F. citri* and bacterial isolate; CK, control; FC, *F. citri*; PA, *Pantoea agglomerans* MX28; FC+PA, *F. citri* plus MX28; M, maculosin; FC+M, *F. citri* plus maculosin. (f–g). Field symptoms of alfalfa leaf blight and symptom development on detached leaves after inoculation with *F. citri* at 0, 3, 5, and 7 days. (h–k). Morphological characteristics of *F. citri*, including conidia observed by light microscopy, and hyphae, macroconidia, and microconidia observed by scanning electron microscopy. (l–n). Lesion diameter, disease incidence, and disease index at 3, 5, and 7 days after inoculation, showing progressive disease development over time. (o). Multilocus phylogenetic analysis based on ITS, *TEF1-α*, and *RPB2* sequences showing placement of the isolate within the *Fusarium citri* clade. Error bars in (l–n) indicate mean ± standard error from five biological replicates. Asterisks indicate statistically significant differences at $P < 0.05$.

Figure 1

See image above for figure legend.

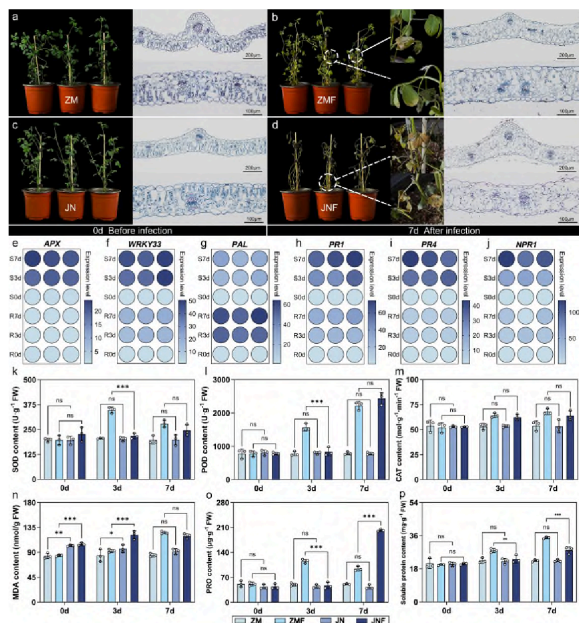


Figure 2. Resistant and susceptible alfalfa cultivars show distinct anatomical, transcriptional, and physiological responses to leaf blight infection. (a–d). Phenotypes, enlarged symptom views, and leaf cross-sections of Zhongmu 3 (ZM), infected Zhongmu 3 (ZMF), Juneng 7 (JN), and infected Juneng 7 (JNF) before infection and at 7 days after infection. ZMF showed milder symptoms and less tissue disruption than JNF. Scale bars in leaf sections are 200 micrometers in the upper images and 100 micrometers in the lower images. **(e–j).** Relative expression of APX, WRKY33, PAL, PR1, PR4, and NPR1 in resistant and susceptible samples at 0, 3, and 7 days after infection. Darker blue circles indicate higher relative expression levels. S0d, S3d, and S7d denote susceptible samples at 0, 3, and 7 days after infection, respectively; R0d, R3d, and R7d denote resistant samples at 0, 3, and 7 days after infection, respectively. **(k–p).** Superoxide dismutase, peroxidase, catalase, malondialdehyde, proline, and soluble protein contents in ZM, ZMF, JN, and JNF at 0, 3, and 7 days after infection. Dots represent individual biological replicates. Error bars indicate mean $\pm$ standard deviation from three biological replicates. Asterisks indicate statistically significant differences at $P < 0.05$, and “ns” indicates no significant difference.

Figure 2

See image above for figure legend.

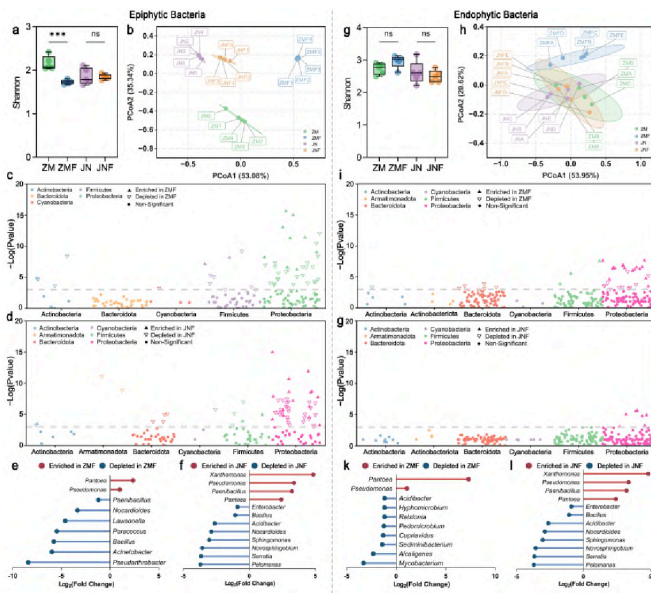


Figure 3. Epiphytic and endophytic bacterial communities differ between resistant and susceptible alfalfa cultivars after infection. (a–d). Alpha diversity, principal coordinate analysis, and differential amplicon sequence variant abundance of epiphytic bacterial communities in Zhongmu 3 (ZM), infected Zhongmu 3 (ZMF), Juneng 7 (JN), and infected Juneng 7 (JNF). (e–f). Differentially abundant epiphytic bacterial genera in ZMF and JNF. Red points and lines indicate enriched genera, and blue points and lines indicate depleted genera. (g–j). Alpha diversity, principal coordinate analysis, and differential amplicon sequence variant abundance of endophytic bacterial communities in ZM, ZMF, JN, and JNF. (k–l). Differentially abundant endophytic bacterial

genera in ZMF and JNF. Red points and lines indicate enriched genera, and blue points and lines indicate depleted genera. Boxes in (a) and (g) show the median and interquartile range, with whiskers indicating the full range of values; dots represent individual biological replicates. Asterisks indicate statistically significant differences at $P < 0.05$, and "ns" indicates no significant difference.

Figure 3

See image above for figure legend.

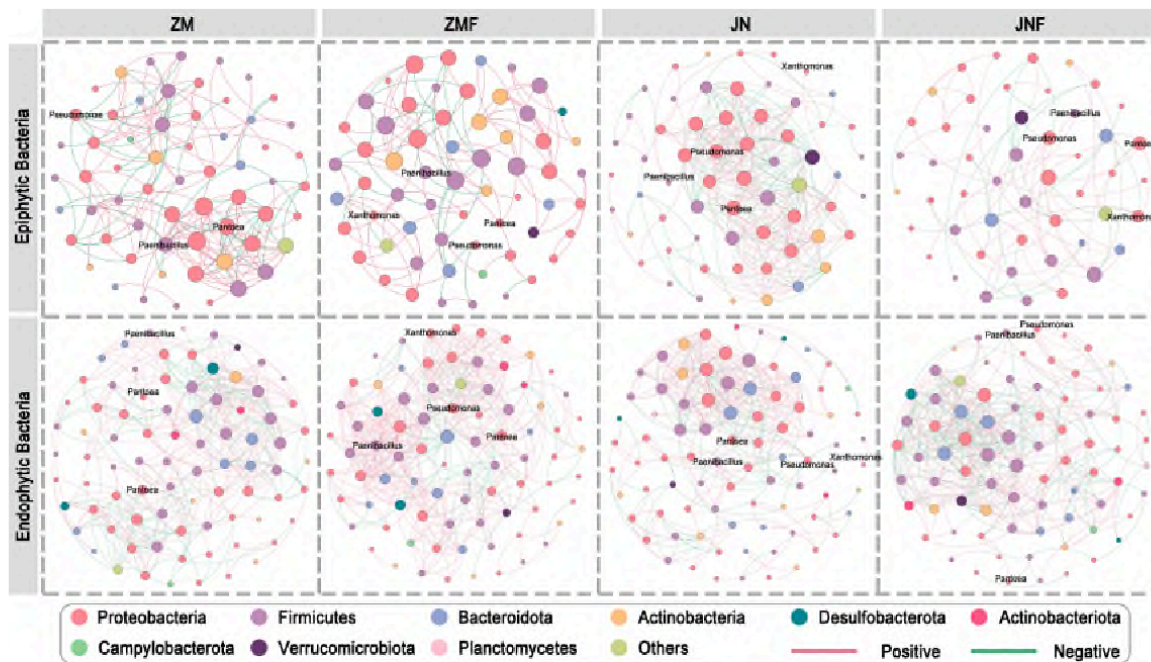


Figure 4. Co-occurrence networks of epiphytic and endophytic bacterial communities in resistant and susceptible alfalfa cultivars after infection. (a–d). Epiphytic bacterial networks in Zhongmu 3 (ZM), infected Zhongmu 3 (ZMF), Juneng 7 (JN), and infected Juneng 7 (JNF). (e–h). Endophytic bacterial networks in ZM, ZMF, JN, and JNF. Nodes represent bacterial taxa, node size indicates relative abundance, edges indicate significant associations, node colors indicate phyla, pink edges indicate positive associations, and green edges indicate negative associations. Selected genera are labeled.

Figure 4

See image above for figure legend.

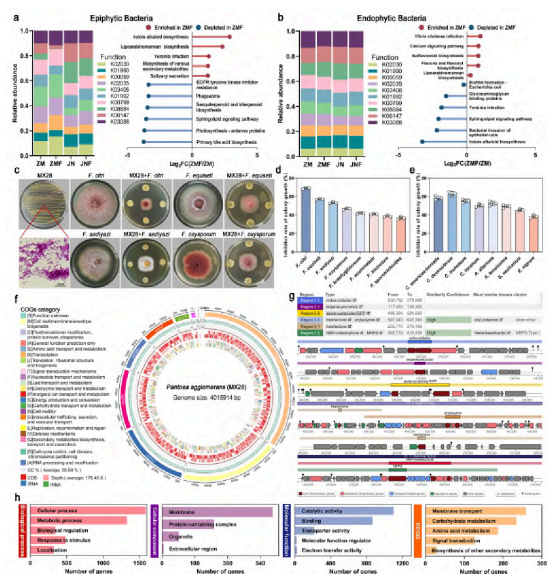


Figure 5. Functional shifts in leaf bacterial communities and characterization of the resistance-associated antagonist *Pantoea agglomerans* MX28. (a,b). Predicted functional profiles of epiphytic and endophytic bacterial communities in Zhongmu 3 (ZM), infected Zhongmu 3 (ZMF), Juneng 7 (JN), and infected Juneng 7 (JNF), and differentially enriched or depleted functions in ZMF relative to ZM. Stacked bars show the relative abundance of functional categories, and dot plots show functions enriched or depleted in ZMF. (c). Colony morphology of MX28, dual-culture assays showing inhibition of *Fusarium citri*, *F. equiseti*, *F. andiyazi*, and *F. oxysporum* by MX28, and microscopic view of MX28 cells. (d,e). Inhibition rates of MX28 against different *Fusarium* species and additional fungal pathogens. (f). Circular genome map of *P. agglomerans* MX28, showing coding sequences, COG functional categories, GC content, and transfer RNA and ribosomal RNA genes. (g). Predicted biosynthetic gene clusters in the MX28 genome, including regions annotated as redox-cofactor, terpene, azole-containing ribosomally synthesized and post-translationally modified peptide, hserlactone, aryl polyenes, and non-ribosomal peptide synthetase-related clusters. (h). Functional annotation of MX28 genes based on Gene Ontology and KEGG categories, including biological process, cellular component, molecular function, and metabolic pathway assignments.

Figure 5

See image above for figure legend.

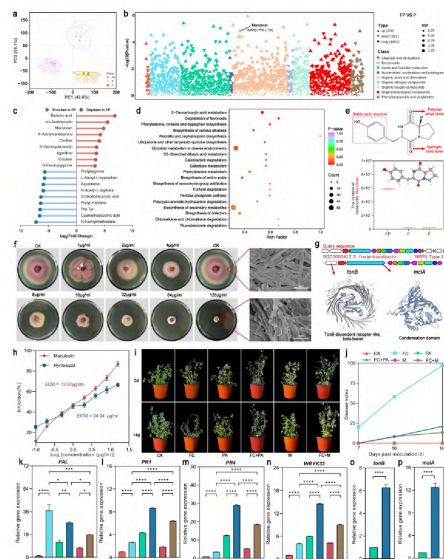


Figure 6. Metabolomic and functional analyses identify maculosin as an antifungal metabolite associated with MX28-mediated disease suppression. (a,b). Principal component analysis and differential metabolite profiles of *Pantoea agglomerans* MX28, *Fusarium citri*, and their co-culture. In (a), points represent MX28 alone (P), *F. citri* alone (F), and co-culture (FP). In

(b), differential metabolites in FP versus P are grouped by chemical class; upward triangles indicate increased metabolites, downward triangles indicate decreased metabolites, and dot size indicates variable importance in projection. (c,d). Differential metabolites and enriched metabolic pathways associated with the co-culture treatment. In (c), red points indicate metabolites enriched in FP and blue points indicate metabolites depleted in FP. In (d), dot size indicates metabolite number and color indicates enrichment significance. (e-h). Chemical structure, relative content, antifungal activity, and putative biosynthetic basis of maculosin. (e) shows the chemical structure and relative content of maculosin in FP, P, and F. (f) shows dose-dependent inhibition of *F. citri* colony growth by maculosin and corresponding scanning electron microscopy images of hyphae under control and maculosin treatment. (g) shows the predicted biosynthetic gene cluster region and structural models of the encoded TonB-dependent receptor-like beta-barrel protein and condensation domain protein. (h) shows the inhibitory activities of maculosin and hymexazol against *F. citri*, with half-maximal effective concentration values indicated. (i,j). Disease phenotypes and disease index of alfalfa plants under the indicated treatments at 0 and 14 days after inoculation. CK, control; FC, *F. citri*; PA, MX28; FC+PA, *F. citri* plus MX28; M, maculosin; FC+M, *F. citri* plus maculosin. (k-p). Relative expression of *PAL*, *PR1*, *PR4*, *WRKY33*, *tonB*, and *mclA* under the indicated treatments.

Figure 6

See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)
- [Additionalfile2.xlsx](#)
- [GraphicalAbstract.docx](#)